TESE DE DOUTORAMENTO

NEW STRATEGIES FOR THE WASTEWATER TREATMENT WITH FILTRATION MEMBRANES

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New strategies for the wastewater treatment with filtration membranes

D. Álvaro Silva Teira

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New strategies for the wastewater treatment with filtration membranes

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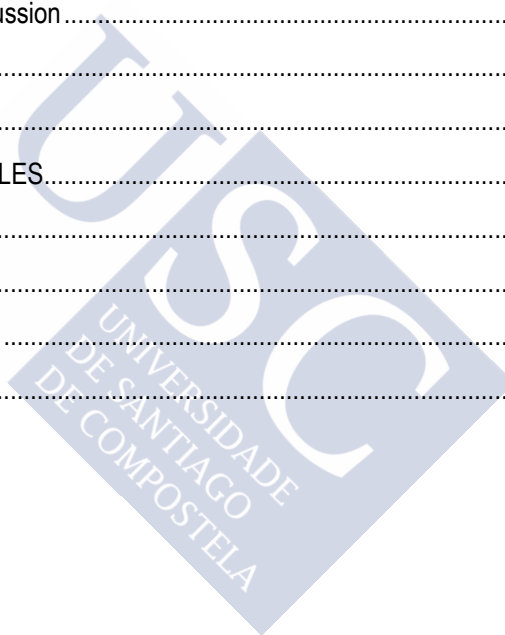En Santiago de Compostela, 28 de setembro de 2018

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OBJECTIVES AND SUMMARY

As a general overview, this PhD thesis focused on wastewater treatment, in both industrial and municipal fields, with a common thread: biomass and treated effluent were separated through an ultrafiltration membrane (10^{-7}-10^{-8} m pore size), substituting the traditional secondary clarifiers.

From 60's onwards, an increasing interest in filtration membranes as mixed liquor/permeate separation systems was detected. Although low efficiencies and high costs were observed in the early stages of membrane bioreactor (MBR) technology, research outputs enhanced an increase of the competitiveness of these technologies in comparison to conventional systems. The application of the scientific knowledge gained in the nineties and 2000s led to a worldwide tremendous growth in the number of full-scale MBR references for treating both sewage and industrial wastewaters. New membrane based alternatives as compact hybrid MBR systems, combining both flocculent and attached biomass, largely increased the treatment capacity of these anoxic/aerobic systems in comparison to MBRs and, especially, with regard to traditional CAS systems. Other approaches, combining anaerobic and MBR treatment stages were launched. These new systems, were able to take profit of the most known advantages, enriched methane biogas generated during the anaerobic decomposition of organic matter, overtaking the typical drawbacks of these systems such as the poor effluent quality, due to the presence of remaining organic matter, in terms of soluble and particulate matter, and the lack of nitrogen elimination. In this way, combining a first anaerobic roughing stage and a second MBR stages, coupled in series, permitted to obtain a high-quality reclaimed effluent, in addition to the above-mentioned biogas.

On the other hand, a drastic reduction of CAPEX and OPEX of MBR based WWTPs was experimented in recent years. MBR systems became competitive alternative respect with CAS and tertiary filtration systems, especially if water reuse of the reclaimed effluent is needed or the treated wastewater is discharged to a sensitive area. In other particular cases, alternative technologies different from MBRs are normally more economically advisable.

CHAPTER 1: Introduction

In this chapter information regarding the main applications, operational and capital expenses, and a bibliometric study is shown. The use of micro (MF) or ultrafiltration (UF) membranes in MBR makes possible to obtain high quality reclaimed effluent. This fact facilitates water reuse either in the industrial field with purposes such as industrial recycling and reuse, for cooling water, boiler feed or process water, or in the municipal water reuse for agricultural or landscape irrigation. A much lower footprint per volume of treated water is occupied by MBRs, typically one third when compared to a conventional activated sludge (CAS) system. This fact encouraged environmental engineers to propose these treatments when an enlargement of an existing wastewater treatment plant (WWTP) was needed in a limited land area. Both characteristics, high-quality effluents or the implementability in places in which the land scarcity is an issue, could be a key factor encouraging decision makers to build-up MBR-based WWTP, especially in countries with a high environmental awareness and/or hydric stress. As a consequence, an annual growth rate of 12.8% was reliably predicted in the period 2014-2019 in the urban field.

In the industrial sector, MBR-based systems have been also growing. As the treated water quality allowed to a water reuse, companies studied the implementability of these systems with a double purpose: decreasing the water pressure in the location surroundings and enhancing their public image. WC flushing or vehicles washing were typical uses of reclaimed water. Besides, the installation of anaerobic MBRs has been a typical option which allowed to gain bioenergy besides the aforementioned reclaimed water. In this way, energy and water invoices were significantly decreased for the company. Both advantages permitted to recover the investment cost in a period as short as 3-4 years in many cases.

Nevertheless, some bottlenecks slowed-down a quicker expansion of MBR market. Capital and operational expenses (CAPEX and OPEX, respectively) have been typically higher in comparison to traditional CAS systems. With regards to CAPEX, filtration membranes and a more complex automatization significantly increased the initial investment. On the other hand OPEX, those costs were drastically related to the aeration demands to counteract the membrane fouling. This phenomenon has been defined as the deposition of solid particulates onto the membrane surface which may

decrease the filtration capacity. By intensively blowing in the membrane surface, fouling can be decreased, promoting both lifespan and membranes' capacity. Although OPEX are still slowly higher when an MBR is present in comparison to traditional systems, recent publications pointed that the difference between MBR and CAS has been decreasing in the last years especially if a tertiary treatment (sand filters and/or UV setups) is installed with views to a water reuse. Thus, MBR-WWTP operated at a cost as low as 0.22 €/m^3 in a well-optimized facility can face to face compete against an average of 0.18 €/m^3 CAS + tertiary WWTP in the municipal sector. These newly launched economic data open the door to a fair competition between the traditional treatments and the MBRs when the treated water is addressed to a further reuse. Hence, the increase of MBRs competitiveness should be necessarily addressed by an enlargement of these MBRs' capacity and/or an improvement of the energetic efficiency in the wastewater treatment.

Regarding the industrial sector, wastewater outputs have been typically much more polluted than the municipal sewage, especially in terms of organic carbon. For this reason, OPEX in industrial wastewater treatment plants (iWWTP) have been normally much higher than those related to municipal water, independently of the installed technology. With this information, two points have been detected to keep improving the MBR-competitiveness in the industrial sector. Energetic expenses, as in the case of the municipal sector. On the other hand, the potential conversion of the organic carbon content into methane-rich biogas. Anaerobic treatments combined with MBRs can allow obtaining bioenergy (profitable as heat or electricity) and reclaimed water, both items reusable for many purposes internally in the same factory. This energetic profit and the decrease of the external hydric pressure leads to a positive economic balance. In this way, this PhD thesis collects the above-summarized information as a whole to cover the detected weak points in the wastewater treatment with membranes in both municipal and industrial sectors.

A bibliometric study regarding the number of papers published per year or the number of patents granted, was carried out and compared with the scientific development in related fields. The results of the study pointed out a decrease in the attractiveness of the MBR research field, with lower rates than expected. Listed below are the briefly

overviewed the contents of each chapter including the focus of the research as long as the main outputs collected from the experimental work.

CHAPTER 2: Materials and methods

Chapter 2 includes the general materials and methods employed during the different experiments performed in the subsequent chapters. An exhaustive description and the references in which each method appeared are also included. This chapter is not original, and was included with the purpose of facilitating information to other researchers, regarding the main analytical methods used in our laboratories during most of the experiments.

CHAPTER 3: Performance of a hybrid membrane bioreactor treating a low strength and alkalinity wastewater

Chapter 3 proposed a hybrid-MBR for enlarging the capacity of an existing WWTP based on a CAS technology. This alternative combines two compact approaches, biofilms, and MBRs, to increase both the eliminated organic and nitrogen loading rates.

Primary settled municipal wastewater was treated in a pilot plant located in an urban WWTP. The system consisted of an anoxic compartment in presence of suspended biomass, a biofilm chamber in which both biofilm and suspended biomass coexist and a membrane compartment (flat sheet ultrafiltration membrane) in which the separation of biomass/treated effluent took place. The system was operated at a competitive hydraulic retention time (HRT) as low as 6 h, on average. The wastewater was characterized by a strongly variable COD_T/TN ratio along the day. The pilot plant stably eliminated solids and organic carbon in an average percentage of 100 and 90%, respectively. Nitrogen removal was fairly limited to 49%. Although ammonium was fully nitrified, denitrification was the bottleneck in nitrogen elimination. One fact which limited denitrification was the scarce BOD_5/TN ratio, especially in valley stages where this value was as low as 2 $mgBOD_5/mgTN$, shortening the denitrification capacity. Moreover, a mechanical limitation of the recycling pump, from the biofilm to the anoxic compartments, could affect to the limited nitrogen removal value in the stages when BOD_5/TN ratio was enough to denitrify. Nitrification consumes twice alkalinity than denitrification releases. As nitrification fully proceeded and denitrification was fairly

limited, this fact together with an inherent wastewater low alkalinity led to pH drops in the final effluent down to 4.5. Despite the extreme conditions for a mixed liquor/effluent separation, membrane performance indicators have been remarkable. Permeability averaged as high as 201 L/(m²·h·bar) when the flux was set at 20 L/(m²·h). Steady-state behavior of the described system could be accurately predicted by the simulator Biowin.

CHAPTER 4. Assessment of a combined UASB and MBR process treating wastewater from a seafood industry at different temperatures

Largely polluted wastewaters generated during the productive processes, high energy invoices and severe water demands are common issues for many food processing industries.

Chapter 4 suggested a combined anaerobic UASB and MBR (2 stages UASB-MBR) for treating the polluted water outputs from a seafood industry at bench scale. This chapter proposed an anaerobic treatment addressed to obtain methane-rich biogas profitable in the same factory, decreasing the external natural gas demands. Moreover, an aerobic MBR coupled in series provided the quality characteristics to the treated water for a further reuse in applications such as washing vehicles or WC flushing or cooling towers, diminishing the hydric pressure in the location's surroundings. The temperature in the anaerobic stage was changed (17-35 ºC) to assess its effect on the system as a whole. The system has been capable of stably removing COD and solids in an average of 94% and 100%, respectively. Optimal performances were achieved at a temperature of 30 ºC set in the UASB reactor with methanation percentages up to 90%. Moreover, the polishing MBR was able to counteract the COD overloads when the anaerobic performances were low. This fact warranted the fulfillment of the discharge limits during the whole experimental period. The presence of quaternary ammonium compounds (QAC) in the wastewater coming from sterilization and cleaning tasks, dramatically inhibited the biological processes in both the anaerobic UASB and the aerobic MBR stages. This fact indicated that their presence should be accurately managed in a full-scale facility to prevent inhibition issues. The membrane filtration process (hollow fiber membrane) behaved remarkably during the first stage of the research with permeability values as high as 356 L/(m²·h·bar). Nevertheless, an automatization malfunction addressed to a membrane operation above the

recommendations and a dramatic decrease in the permeability was observed to values down to 90 L/(m^2·h·bar).

CHAPTER 5. Removal of dissolved methane and nitrogen from anaerobically treated effluents at low temperature by MBR post-treatment

Chapter 5 proposed an innovative system to foster a well-known issue when dealing with anaerobic systems, especially when they are operated at low temperatures, the methane dissolved in the liquid stream. Methane has been considered a strong greenhouse gas (GHG) with a warming potential 34-times higher than CO_2. Methane content present in the gas stream of an anaerobic reactor is easily controllable and valuable. Nevertheless, if this compound is present in the liquid phase, it will be stripped off due to the turbulence generated due to the liquid stream circulation or in the coupled-in-series polishing reactor. For this reason, dissolved methane should be eliminated before the effluent leaves the system. At the end of the past century, microorganisms able to employ this gas as an inexpensive electron donor to denitrify has been discovered. In this chapter, a system is proposed to take advantage of this newly discovered microbiological process to correct the wastewater nitrogen concentration by using methane as electron donor in denitrification. In this way, a double goal was aimed, on the one hand, to decrease the nitrogen concentration of the treated water, with views of widening its applicability in a further reuse, and, to reduce environmental impacts related to the dissolved methane. A bench scale combined reactor featured in a UASB (anaerobic stage) coupled in series to an anoxic/aerobic MBR (with an ultrafiltration hollow fiber membrane) was operated to assess the performance indicators. The system was fed with dairy low strength wastewater. The overall system UASB + MBR has been operated at an average HRT of 15 h. COD and TSS were eliminated in a 95 and 100%, respectively. As dissolved methane in the liquid effluent of the UASB reactor ranged between 20-30% of the overall methane generated, the MBR post-treatment consistently eliminated 80% of this dissolved methane. Nitrification fully proceeded in the aerobic chamber of the MBR and up to 15 mgTN/L was denitrified. Although mass balances indicated that a fraction of the eliminated methane was still unknown, it was demonstrated that the denitrification with methane proceeded in this reactor. Microbiological representatives were found by employing the FISH technique and batch assays confirmed the

denitrification with methane. Regarding membrane performance indicators relatively high values were reached with a permeability ranged between 100 and 250 L/m^2·h·bar.

CHAPTER 6. Overview of the competition among oxidizers of methane and ammonium in an oxygen scarcity environment

In Chapter 5, the main unknown point detected on the overall process has been the lack of knowledge in the complex microbiological processes involved in the combined elimination of both, methane and nitrogen. This fact encouraged to continue the research on this topic in a center specialized in these interactions. As a consequence, Chapter 6 intensively focused on gaining further information about the interaction of nitrogen and methane cycles which in turn can be applied in a further optimization of the system presented in Chapter 5. Chapter 6 has been fully held in the Department of Microbiology of Radboud Universiteit Nijmegen (The Netherlands), recognized around the world by its research targeted in knowing the key players in both nitrogen and methane cycles in oxic/anoxic environments.

The first tasks performed in this Chapter have been referred to the enrichment of ammonium oxidizing bacteria (AOB) and methane-oxidizing bacteria (MOB). In the case of AOBs, an enrichment strategy in two coupled in series combined continuous reactors (Reactors A and B; 2 L each) was experimented. Alternatively, MOB were enriched by means of discontinuous cultures. In the first case, A reactor was operated at conditions to promote AOB (oxygen scarcity, low SRT governed by a withdrawal stream) but nitrite accumulation was not detected in any point of the experimental period. As an output of the performed PCR based tests, AOB were undoubtedly present but the absence of nitrite led to think that other populations were present in the medium (latter newly discovered complete ammonium oxidation organisms, commamox, populations have been detected). Thus, a discontinuous enrichment strategy displaced the previous continuous experiments aimed at collecting a big number of AOB representatives. A pure culture of *Nitrosomonas europaea* was selected to be enriched by their representatives. After some weeks of harvest, nitrite strips indicated that AOB were largely present. *Methylomonas lenta* discontinuous harvests were successful in the first trial.

The second part of the research held in Chapter 6 was the search of:

Nitrosomonas europaea:

I) The inhibitory capacity of methane (MOB substrate) in the AOB activity. The experiments indicated that AOB could handle a 5% methane in the headspace at maximum. Larger concentrations totally inhibited the oxidizing capacity of AOBs.

II) Methane as an alternative substrate for the AOB. Previously, it was reported that when ammonium was absent in the medium, methane could be employed as a substrate for the oxidizing activity of AOB. As an output of this experiment, if the headspace methane concentration accounted up to 10%, AOB could oxidize methane, demonstrating AOB capacity for employing methane as substrate. Nevertheless, if methane concentration increased up to 20% the oxidizing capacity was only observed in the first 135 min of experimentation, indicating that a poisoning effect on the AOB representatives was detected. Thus, methane as a substrate was only a stable alternative at low concentrations.

Methylomonas lenta:

III) The inhibitory threshold of ammonium in MOB activity. Although a poisoning effect was not observed in any of the ammonium concentrations experimented (0-520 μM NH_4^+), the higher concentration of this compound the lower methane oxidation capacity was observed. Alternatively, when ammonium was absent and only methane was present in the medium as a substrate, it was not detected any oxidation of ammonium. It demonstrated the incapacity of MOB for employing ammonium as a substrate, in contrast to AOB capacity of using methane an alternative substrate.

IV) The inhibitory threshold of nitrite in MOB activity. As nitrite could be present in a further experiment of the competition of MOB and AOB, it was studied its influence on MOB activity. It was found that depending on its concentration in the medium it could either enhance (99-250 μM NO_2^-) or inhibit (1000 μM NO_2^-) the MOB oxidative process.

Lastly, biological oxygen monitor (BOM) assays were performed to assess the minimum concentration of dissolved oxygen required to carry out the oxidizing activities of both *Nitrosomonas europaea* and *Methylomonas lenta*. In both cases, it

was found that it was not required a minimum dissolved oxygen concentration to start the biological activity, indicating that a minimum oxygen threshold was not needed to perform the biological oxidation.

OBJETIVOS Y RESUMEN

Como resumen general, esta tesis doctoral se centró en el tratamiento de aguas residuales, tanto en el ámbito industrial como municipal, con un mismo hilo conductor: los tradicionales clarificadores secundarios fueron sustituidos para la separación de la biomasa y el efluente tratado que se separaron a través de una membrana de ultrafiltración (10^{-7}-10^{-8} m de tamaño de poro).

Desde los años 60 en adelante, se detectó un interés creciente en las membranas de filtración como sistemas de separación entre licor mezcla/permeado. En las primeras referencias de la tecnología de biorreactor de membranas (BRM), los resultados de la actividad investigadora han conseguido aumentar la competitividad de estas tecnologías en comparación con los sistemas convencionales. La aplicación del conocimiento científico adquirido en los años noventa y 2000 dio lugar a un enorme crecimiento mundial en el número de referencias de BRM a gran escala para el tratamiento de las aguas residuales, tanto residuales como industriales. Las nuevas alternativas basadas en membranas, como los sistemas BRM híbridos compactos, que combinan biomasa asociada y floculante, aumentaron en gran medida la capacidad de tratamiento de estos sistemas anóxicos/aerobios en comparación con los BRM simples y, especialmente, con respecto a los sistemas de lodos activados convencionales. En esta época se lanzaron otras alternativas, combinando etapas de tratamiento anaeróbico y BRM. Estos nuevos sistemas, han aprovechado las ventajas más conocidas de los sistemas metanogénicos, como la producción de biogás con alto contenido en metano, generado durante la descomposición de la materia orgánica; y superando los inconvenientes tradicionales de estos sistemas, como la mala calidad del efluente, debido a la presencia de materia orgánica remanente, en términos de materia soluble y particulada, y la falta de eliminación de nitrógeno. De esta forma, la combinación de una primera etapa de degradación anaerobia y la segunda etapa de BRM, acopladas en serie, permitió obtener un efluente recuperado de alta calidad, además del anteriormente mencionado biogás.

Por otro lado, en los últimos años, se experimentó una reducción drástica de los gastos de capital, siglas en inglés CAPEX, y gastos de operación, del inglés

OPEX, de las EDAR basadas en BRM. Los sistemas BRM se han convertido en una alternativa competitiva con respecto a los sistemas combinados de fangos activos con tratamiento terciario, especialmente si se necesita la reutilización del agua tratada o si el agua residual tratada se descarga en un área sensible. En otros casos, tecnologías diferentes a los BRM normalmente son económicamente más fiables.

CAPÍTULO 1: Introducción

En este capítulo se muestra una recopilación de datos sobre las principales aplicaciones, gastos operativos y de capital, y un estudio bibliométrico actualizado a los últimos años. El uso de membranas micro (MF) o de ultrafiltración (UF) en BRMs permite obtener efluentes recuperados de alta calidad. Este hecho facilita la reutilización del agua en el campo industrial con fines tales como el reciclaje y la reutilización industrial, para el agua de refrigeración, de alimentación de calderas o agua de proceso, o, alternativamente, en la reutilización del agua municipal para riego agrícola o paisajístico. Los BRM ocupan una superficie de terreno mucho menor por volumen de agua tratada, generalmente un tercio, en comparación con un sistema convencional de lodos activados. Este hecho alentó a ingenieros ambientales a proponer estos tratamientos en los casos de una ampliación de una planta de tratamiento de aguas residuales (EDAR) existente o cuando la disponibilidad de suelo era limitada. Ambas características, los efluentes de alta calidad o la implementabilidad en lugares donde la escasez de tierra es una realidad, puede ser un factor clave que motive a construir EDARs basadas en BRM, especialmente en países con una alta conciencia ambiental y/o estrés hídrico. Como consecuencia, se ha pronosticado de manera fiable una tasa de crecimiento anual del 12,8% en el periodo 2014-2019 en el ámbito urbano.

En el sector industrial, los sistemas basados en BRM también han crecido. La calidad del agua tratada ha permitido la reutilización del agua, por lo que las empresas estudiaron su aplicabilidad con un doble propósito: disminuir la presión hídrica en los alrededores de la ubicación de la fábrica, y la mejora de su imagen pública. La descarga de inodoros o el lavado de vehículos han sido usos típicos del agua recuperada en instalaciones industriales. Además, la instalación de BRM anaerobios ha sido una opción típica que permitió la obtención de bioenergía y

agua regenerada. De esta forma, las facturas de energía y agua se redujeron significativamente en la cuenta de resultados de la empresa. Ambas ventajas permitieron recuperar el coste de inversión en un breve período de 3-4 años en muchos casos.

Sin embargo, se han detectado algunos cuellos de botella que atrasaron una expansión más rápida del mercado de los BRM. Los gastos operativos y de capital (CAPEX y OPEX, respectivamente) han sido comúnmente más altos en comparación con los sistemas de lodos activos tradicionales. Con respecto a los CAPEX, las membranas de filtración y una automatización más compleja aumentaron significativamente la inversión inicial. Por otro lado, los OPEX se relacionaban drásticamente con las demandas de aireación para contrarrestar el ensuciamiento de la membrana. Este último fenómeno, definido como la deposición de partículas sólidas sobre la superficie de la membrana, puede disminuir la capacidad de filtración. El burbujeo intenso sobre la superficie de la membrana disminuye el ensuciamiento, incrementando tanto la vida útil como la capacidad de las membranas. Aunque los OPEX son más bajos cuando hay un sistema de lodos activados en comparación con los BRM, publicaciones recientes indican que la diferencia entre BRM y lodos activados ha disminuido en los últimos años, especialmente si se instala un terciario (filtros de arena y/o configuraciones de UV) tras el tratamiento secundario tradicional, con vistas a una reutilización de agua. Ya se ha reportado que EDAR basadas en BRM han operado a un coste tan bajo como 0,22 €/m^3, en instalaciones bien optimizadas, este hecho hace que BRM puedan competir contra sistemas de lodos activados con costes reportados promedio de 0,18 €/m^3 para las combinaciones de tradicional secundario y terciario en el sector municipal. Estos datos económicos recientemente aparecidos abren la puerta que BRM y lodos activos puedan competir cuando el agua tratada se destina a una reutilización. Por este motivo, la competitividad de los BRM debería enfocarse necesariamente a los casos de una ampliación de la capacidad de estos BRM y/o una mejora de la eficiencia energética en el tratamiento de aguas residuales.

Con respecto al sector industrial, sus aguas residuales han resultado ser normalmente mucho más contaminadas que las aguas residuales municipales,

especialmente en términos de carbono orgánico. Por esta razón, los OPEX en las plantas de tratamiento de aguas residuales industriales (EDARi) han sido normalmente mucho más altos que los relacionados con el agua municipal, independientemente de la tecnología instalada. Con esta información, se han detectado dos puntos de mejora de eficiencia de los BRM en el sector industrial. Por un lado, los consumos energéticos. Por otro, la conversión del contenido de carbono orgánico en biogás. Los tratamientos anaeróbicos combinados con BRM permiten la obtención de bioenergía, valorizable como calor o electricidad, y agua regenerada, reutilizables para muchos fines internamente en la propia fábrica. La valorización energética y la disminución de la presión hídrica externa conducen a inversión rápidamente retornable. De esta forma, esta tesis doctoral recoge la información anteriormente resumida para superar los puntos débiles detectados en el tratamiento de aguas residuales con membranas en los sectores municipal e industrial.

CAPÍTULO 2: Materiales y métodos

El Capítulo 2 incluye los materiales y métodos generales empleados en las investigaciones recogidas durante los diferentes capítulos experimentales. También se recoge una descripción exhaustiva de los mismos y las referencias en las que se publicó cada método. Este capítulo no es original y se incluyó con el propósito de facilitar información a otros investigadores con respecto a los principales métodos analíticos utilizados en nuestros laboratorios durante la mayoría de los experimentos.

CAPÍTULO 3: Aguas residuales municipales tratadas por un biorreactor compacto híbrido de membranas

El Capítulo 3 propuso un BRM híbrido para la ampliación de la capacidad de una EDAR existente basada en una tecnología de lodos activos. Esta alternativa combina dos estrategias para conseguir hacer más compacta la depuradora, biopelículas y BRM. Así se podrán aumentar las cargas contaminantes orgánica y de nitrógeno eliminadas.

Las aguas residuales urbanas procedentes de decantación primaria primarias se trataron en una planta piloto ubicada en una EDAR municipal. La configuración implementada en el prototipo consistió en un compartimento anóxico con biomasa suspendida, una cámara de aerobia biofilm en la que coexisten biofilm y biomasa suspendida y un compartimento de membrana (ultrafiltración de placa plana) donde tuvo lugar la separación de la biomasa/efluente tratado. El sistema fue operado a un tiempo de retención hidráulica (TRH) muy competitivo de tan solo 6 h de promedio. Las aguas residuales se caracterizaron por una relación muy variable de la relación DQO_T/NT a lo largo del día. La planta piloto eliminó de manera estable sólidos y carbono orgánico en un porcentaje promedio entre 90 y 100%, respectivamente. La eliminación de nitrógeno estuvo limitada al 49%. Aunque el amonio se ha nitrificado completamente, la desnitrificación fue el cuello de botella en la eliminación de este nutriente. Un hecho que limitó la desnitrificación fue la escasa relación DBO_5/NT, especialmente en las etapas valle del día donde este valor de solo 2 mg $DBO_5/mgNT$, limitando la capacidad de desnitrificación. Además, un la limitación de la capacidad de bombeo de la bomba de recirculación, desde la cámara de biopelículas hasta la anóxica, podría haber limitado también la eliminación de nitrógeno en las etapas en las que la relación DBO_5/NT era suficiente para desnitrificar. La nitrificación consume dos veces la alcalinidad que desnitrificación libera. Como la nitrificación se llevó a cabo completamente y la desnitrificación era limitada, este hecho, junto con una baja alcalinidad inherente del agua residual, condujo a una disminución del pH en el efluente final por debajo de 4.5. A pesar de estas condiciones extremas para una separación de licor mezcla/efluente, los indicadores de rendimiento de la membrana han sido sobresalientes. La permeabilidad promedió 201 L/(m^2·h·bar) cuando el flujo se ajustó a 20 L/(m^2·h). El simulador Biowin ha predicho un comportamiento similar al del sistema real.

CAPÍTULO 4. Evaluación de un proceso combinado de UASB y BRM que trata aguas residuales de una industria de procesado de productos del mar a diferentes temperaturas

Las aguas residuales altamente contaminadas generadas durante los procesos productivos, las facturas derivadas de los altos consumos energéticos y las

severas demandas de agua son problemas comunes para muchas industrias de procesamiento de alimentos.

El Capítulo 4 sugirió una combinación de reactores anaeróbico UASB y BRM de dos etapas en serie para tratar el agua contaminada de una industria de manufactura de productos marinos. Este capítulo ha propuesto un tratamiento anaeróbico, para la transformación de la contaminación orgánica en biogás rico en metano, valorizable en la misma fábrica y disminuyendo las demandas externas de gas natural. Además, un BRM aeróbico acoplado en serie que proporcionó las características de calidad de agua tratada para su posterior reutilización en aplicaciones tales como lavado de vehículos, torres de refrigeración o descargas de WC, disminuyendo la presión hídrica en la zona. La temperatura en la etapa anaeróbica se configuró (17-35 ºC) para evaluar su efecto en el sistema. Éste ha sido capaz de eliminar de manera estable DQO y sólidos en un promedio de 94% y 100%, respectivamente. Se lograron rendimientos óptimos a una temperatura controlada de 30 ºC en el reactor UASB con porcentajes de metanización de hasta el 90%. Además, el BRM fue capaz de contrarrestar las sobrecargas de DQO cuando los rendimientos en la etapa anaerobia eran bajos. Ello ha llevado al cumplimiento de los límites de descarga durante todo el período experimental. La presencia de compuestos de amonio cuaternario (QAC) en las aguas residuales provenientes de tareas de esterilización y limpieza, inhibió drásticamente los procesos biológicos en las etapas anaeróbicas UASB y BRM aeróbica. Este hecho indicó que su presencia debe ser gestionada con precisión en una instalación a gran escala para evitar problemas de inhibición. El proceso de filtración (membrana de fibra hueca) se comportó notablemente durante la primera etapa de la experimentación, con valores de permeabilidad tan altos como 356 L/(m^2·h·bar). Sin embargo, un fallo en la automatización llevó a la operación de la membrana por encima de las recomendaciones del fabricante. Ello llevó a una disminución dramática en la permeabilidad a valores de hasta 90 L/(m^2·h·bar).

CAPÍTULO 5. BRM como tratamiento posterior de efluentes tratados anaeróbicamente

El Capítulo 5 propuso un sistema novedoso para paliar un problema muy conocido cuando se trata de sistemas de depuración anaeróbicos, especialmente cuando se operan a bajas temperaturas, el metano disuelto en la corriente efluente. El metano se ha considerado un fuerte gas de efecto invernadero (GEI), con un potencial de calentamiento 34 veces mayor que el CO_2. El contenido de metano presente en la corriente gas de un reactor anaeróbico es fácilmente controlable y valorizable. Sin embargo, si este compuesto está presente en la fase líquida, se eliminará a la atmósfera debido a la turbulencia generada en la circulación de la corriente líquida o en el reactor BRM de adecuación del efluente acoplado en serie. Por esta razón, el metano disuelto debe eliminarse antes de que el efluente salga del sistema. A finales del siglo pasado, se descubrieron microorganismos capaces de emplear este gas metano como donador de electrones sin coste para desnitrificar. En este capítulo, se propone un sistema para aprovechar este proceso microbiológico recién descubierto y aprovechar para corregir la concentración de nitrógeno del agua residual mediante el uso de metano como donador de electrones en la desnitrificación. De esta forma, se pretendía un objetivo doble, por un lado, para disminuir la concentración de nitrógeno del agua tratada, con vistas a ampliar su aplicabilidad en reutilización, y para reducir los impactos ambientales relacionados con el metano disuelto. Se utilizó un reactor a escala bancada formado por un UASB (etapa anaeróbica) acoplado en serie a un BRM anóxico/aerobio (con membrana de fibra hueca de ultrafiltración). El sistema fue alimentado con aguas residuales lácteas de baja concentración. En general, el sistema se ha operado a un TRH promedio de 15 h. DQO y SST fueron eliminados en un 95 y 100%, respectivamente. El metano disuelto en el efluente líquido del reactor UASB varió entre el 20-30% del metano total generado, el post-tratamiento MBR eliminó sistemáticamente el 80% de este metano disuelto. La nitrificación se realizó en la cámara aeróbica del BRM y se consiguió desnitrificar hasta 15 mgNT/L. Aunque los balances de masa indicaron que todavía se desconocía una fracción del metano eliminado, se demostró que la desnitrificación con metano había tenido lugar en este reactor. Se encontraron representantes microbiológicos empleando la técnica FISH y los ensayos en discontinuo este hecho. Con respecto a los indicadores de rendimiento

de la membrana, se alcanzaron valores relativamente altos con una permeabilidad que oscilaba entre 100 y 250 L/(m²·h·bar).

CAPÍTULO 6. Descripción general de la competencia entre los oxidantes de metano y amonio en un entorno de escasez de oxígeno

En el Capítulo 5, la principal complicación detectada en el proceso global ha sido la falta de conocimiento en los complejos procesos microbiológicos involucrados en la eliminación combinada de metano y nitrógeno. Este hecho animó a continuar la investigación sobre este tema en un centro especializado en estas interacciones biológicas. Así, el Capítulo 6 se centró en obtener más información sobre la interacción de los ciclos de nitrógeno y metano, que podría ser aplicado en una optimización adicional del sistema presentado en el Capítulo 5. El Capítulo 6 se ha llevado a cabo en el Departamento de Microbiología de la Radboud Universiteit Nijmegen (Países Bajos), reconocida en todo el mundo por su investigación dirigida a conocer los principales actores en los ciclos de nitrógeno y metano en entornos óxicos y anóxicos.

Las primeras tareas realizadas en este capítulo se han basado en el enriquecimiento de las bacterias oxidantes de amonio (AOB) y las bacterias oxidantes de metano (MOB). En el caso de las AOB, se planteó una estrategia de enriquecimiento en dos reactores continuos combinados en serie (Reactores A y B, 2 L cada uno). Alternativamente, MOB se enriqueció mediante reactores discontinuos. En el primer caso, se hizo funcionar un reactor en condiciones adecuadas para promover la proliferación de AOB (escasez de oxígeno, bajo tiempo de retención de sólidos, TRS, instalando una corriente de purga) pero la acumulación de nitrito no se detectó en ningún momento del período experimental. Como resultado de las pruebas basadas en PCR, las AOB estaban presentes sin ninguna duda, pero la ausencia de nitrito llevó a pensar que otras poblaciones estaban presentes en el medio. Se sospechaba de la presencia de unos microorganismos capaces de oxidar de amonio completamente hasta nitrato recientemente descubiertos, commamox. Posteriormente, otras pruebas han confirmado la presencia de estas poblaciones. Como consecuencia de lo anterior, una estrategia de enriquecimiento discontinua substituyó a los experimentos continuos. Un cultivo puro de *Nitrosomonas europaea* fue seleccionado para ser enriquecido. Después de algunas semanas de cosecha, tiras de nitrito indicaron que

AOB estaba presente en gran medida. Los cultivos discontinuos de *Methylomonas lenta* fueron exitosos ya en el primer ensayo.

La segunda parte de la investigación realizada en el Capítulo 6 fue la búsqueda de:

Nitrosomonas europaea:

I) La capacidad inhibitoria del metano (sustrato MOB) en la actividad AOB. Los experimentos indicaron que las AOB podrían tener actividad con metano de hasta el 5% en el espacio de cabeza. Concentraciones más altas inhibieron totalmente la capacidad oxidante de las AOB.

II) Metano como sustrato alternativo para las AOB. Previamente, se reportó que cuando el amonio no estaba presente en el medio, el metano podría emplearse como sustrato para la actividad oxidante de las AOB. Como resultado de este experimento, si la concentración de metano en el espacio de cabeza representaba hasta el 10%, las AOB podrían oxidar el metano, demostrando la capacidad de AOB para emplear el metano como sustrato. Sin embargo, si la concentración de metano aumentaba hasta el 20%, la capacidad oxidante solo se observó en los primeros 135 min de experimentación, lo que indica que se detectó un efecto de envenenamiento en los representantes de AOB. Por lo tanto, el metano como sustrato era solo una alternativa estable a bajas concentraciones.

Methylomonas lenta:

III) El umbral inhibidor de amonio en la actividad de las MOB. Aunque no se observó un efecto de envenenamiento en ninguna de las concentraciones de amonio experimentadas (0-520 µM NH_4^+), se observó que una mayor concentración de este compuesto ha implicado una menor capacidad de oxidación de metano. Alternativamente, cuando el amonio estaba ausente y solo el metano estaba presente en el medio como sustrato, no se detectó ninguna oxidación de amonio. Así, este hecho, demostró la incapacidad de MOB para emplear amonio como sustrato, en contraste con la capacidad AOB de usar metano como sustrato alternativo.

IV) El umbral inhibidor del nitrito en la actividad de las MOB. Se encontró que dependiendo de su concentración en el medio, podía potenciar (99-250 µM NO_2^-) o inhibir (1000 µM NO_2^-) el proceso oxidativo MOB.

Por último, se realizaron ensayos de monitorización del oxígeno biológico (BOM) para evaluar la concentración mínima de oxígeno disuelto requerida para llevar a cabo las actividades oxidantes tanto de *Nitrosomonas europaea* como de *Methylomonas lenta*. En ambos casos, se encontró que no se requería una concentración mínima de oxígeno disuelto para iniciar la actividad biológica, lo que indica que no se necesitaba un umbral mínimo de oxígeno para realizar la oxidación biológica.

OBXECTIVOS E RESUMO

Como resumo xeral, esta tese de doutoramento centrouse no tratamento de augas residuais, tanto no ámbito industrial como municipal, cun mesmo fío condutor: os clarificadores secundarios tradicionais foron substituídos por unha membrana de ultrafiltración (10^{-7}-10^{-8} m de tamaño de poro) para a separación da biomasa e o efluente tratado.

Desde os anos 60 en diante, detectouse un interese crecente nas membranas de filtración como sistemas de separación entre licor de mestura e permeado. Nas primeiras referencias da tecnoloxía de biorreactor de membranas (BRM) os resultados de eficiencia e custos eran pouco competitivos con respecto aos sistemas convencionais. A aplicación do coñecemento científico adquirido nos anos noventa e 2000 deu lugar a un enorme crecemento mundial no número de referencias de BRM a gran escala para o tratamento das augas residuais, tanto municipais como industriais. As novas alternativas baseadas en membranas, como os sistemas BRM híbridos compactos, que combinaron biomasa adherida e floculenta, aumentaron en gran medida a capacidade de tratamento destes sistemas anóxicos/aerobios en comparación cos BRM simples e, especialmente, con respecto aos sistemas de lodos activados convencionais. Nesta época lanzáronse outras alternativas, combinando etapas de tratamento anaerobio e BRM. Estes novos sistemas, aproveitaron as vantaxes máis coñecidas dos sistemas metanoxénicos, como a produción de biogás con alto contido en metano, xerado durante a descomposición da materia orgánica; e superaron os inconvenientes tradicionais destes sistemas, como a mala calidade do efluente, debido á presenza de materia orgánica remanente, en termos de materia soluble e particulada, e a falta de eliminación de nitróxeno. Desta forma, a combinación dunha primeira etapa de degradación anaerobia e a segunda etapa de BRM, dispostas en serie, permitiu obter un efluente recuperado de alta calidade, ademais do anteriormente mencionado biogás.

Doutra banda, nos últimos anos, experimentouse unha redución drástica dos gastos de capital, siglas en inglés CAPEX, e gastos de operación, do inglés OPEX, das EDAR baseadas en BRM. Os sistemas BRM convertéronse nunha

alternativa competitiva con respecto aos sistemas combinados de lodos activados con tratamento terciario, especialmente se se necesita a reutilización da auga ou si a auga residual tratada se descarga nunha área sensible. Noutros casos, tecnoloxías diferentes aos BRM normalmente son economicamente máis aconsellables.

CAPÍTULO 1: Introdución

Neste capítulo móstrase unha recompilación de datos sobre as principais aplicacións, gastos operativos e de capital, e un estudo bibliométrico actualizado aos últimos anos no campo das membranas de ultrafiltración. O uso de membranas micro (MF) ou de ultrafiltración (UF) en BRMs permite obter efluentes recuperados de alta calidade. Este feito facilita a reutilización da auga no campo industrial con fins tales como a reciclaxe e a reutilización industrial, para a auga de refrixeración, de alimentación de caldeiras ou auga de proceso, ou, alternativamente, na reutilización da auga municipal para rego agrícola ou paisaxístico. Os BRM ocupan unha superficie de terreo moito menor por volume de auga tratada, xeralmente un terzo, en comparación cun sistema convencional de lodos activados. Este feito alentou a enxeñeiros ambientais a propoñer estes tratamentos nos casos dunha ampliación dunha planta de tratamento de augas residuais existente ou cando a dispoñibilidade de solo era limitada. Ámbalas dúas características, efluentes de alta calidade ou a posibilidade de implantación en lugares onde a escaseza de terreo é unha realidade, pode ser un factor crave que motive a construír EDARs baseadas en BRM, especialmente en países cunha alta conciencia ambiental e/ou estrés hídrico. Como consecuencia, prognosticouse de xeito fiable unha taxa de crecemento anual do 12,8% no período 2014-2019 no ámbito urbano.

No sector industrial, os sistemas baseados en BRM tamén creceron. A calidade da auga tratada permitiu a reutilización da auga, polo que as empresas estudaron a súa aplicabilidade cun dobre propósito: diminuír a presión hídrica nos arredores do emprazamento da fábrica, e a mellora da súa imaxe pública. Descargas de inodoros ou lavado de vehículos foron usos típicos da auga recuperada en instalacións industriais. Ademais, a instalación de BRM anaerobios foi unha alternativa que permitiu a obtención de bioenerxía e auga rexenerada. Desta

forma, as facturas de enerxía e auga reducíronse de maneira significativa na conta de resultados da empresa. Ambas vantaxes permitiron recuperar o custo de investimento nun período breve de 3-4 anos en moitos casos.

Con todo, detectáronse algúns puntos de mellora que atrasaron unha expansión máis rápida do mercado dos BRM. Os gastos operativos e de capital (CAPEX e OPEX, respectivamente) foron comunmente máis altos en comparación cos sistemas de lodos activados tradicionais. Con respecto aos CAPEX, as membranas de filtración e unha automatización máis complexa aumentaron de xeito cuantioso o investimento inicial. Doutra banda, os OPEX relacionábanse drasticamente coas demandas de aireación para contrarrestar o ensuzamiento da membrana. Este último fenómeno, definido como a deposición de partículas sólidas sobre a superficie da membrana, pode diminuír a capacidade de filtración. A aireación intensa sobre a superficie da membrana diminúe o ensuzamiento, incrementando tanto a vida útil como a capacidade das membranas. Aínda que os OPEX son máis baixos cando hai un sistema de lodos activados en comparación cos BRM, publicacións recentes indican que a diferenza entre BRM e lodos activados diminuíu nos últimos anos, especialmente en presenza dun tratamento terciario (filtros de area e/ou configuracións de UV) tralo secundario tradicional, con vistas á reutilización de auga. Xa se reportou que EDAR baseadas en BRM operaron a un custo tan baixo como 0,22 €/m³, en instalacións ben optimizadas. Este feito fai que os BRM poidan competir contra sistemas de lodos activados con custos medios de 0,18 €/m³ para as combinacións de secundario tradicional e terciario no sector municipal. Estes datos económicos recentemente aparecidos abren a porta a que BRM e lodos activados poidan competir cando a auga tratada se destina á reutilización. Por este motivo, a competitividade dos BRM debería enfocarse necesariamente aos casos dunha ampliación da capacidade das EDAR e/ou unha mellora da eficiencia enerxética no tratamento de augas residuais.

Con respecto ao sector industrial, as súas augas residuais resultaron ser normalmente moito máis contaminadas que as municipais, especialmente en termos de carbono orgánico. Por esta razón, os OPEX nas plantas de tratamento de augas residuais industriais (EDARi) foron normalmente moito máis altos ca os

relacionados coa auga municipal, independentemente da tecnoloxía instalada. Con esta información, detectáronse dous puntos de mellora de eficiencia dos BRM no sector industrial. Por unha banda, os consumos enerxéticos. Por outro, a conversión do contido de carbono orgánico en biogás. Os tratamentos anaerobios combinados con BRM permiten a obtención de bioenerxía, empregable como calor ou electricidade, e auga rexenerada, reutilizable para moitos fins internamente na propia fábrica. A valorización enerxética e a diminución da presión hídrica externa conducen a investimentos rápidamente retornables. Desta forma, esta tese de doutoramento recolle a información anteriormente resumida para superar os puntos débiles detectados no tratamento de augas residuais con membranas nos sectores municipal e industrial.

CAPÍTULO 2: Materiais e métodos

O Capítulo 2 inclúe os materiais e métodos xerais empregados nas investigacións recollidas nos diferentes capítulos experimentais. Tamén se recolle unha descrición exhaustiva dos mesmos e as referencias nas que se publicou cada método. Este capítulo non é orixinal e inclúese co propósito de facilitar información a outros investigadores con respecto aos principais métodos analíticos utilizados nos nosos laboratorios durante a maioría dos experimentos.

CAPÍTULO 3: Augas residuais municipais tratadas por un biorreactor compacto híbrido de membranas

O Capítulo 3 propuxo un BRM híbrido para a ampliación da capacidade dunha EDAR existente baseada nunha tecnoloxía de lodos activados. Esta alternativa combina dúas estratexias para conseguir facer máis compacta a depuradora, biofilmes e BRM. Así, poderanse aumentar as cargas contaminantes orgánica e de nitróxeno eliminadas.

As augas residuais urbanas procedentes de decantación primaria tratáronse nunha planta piloto situada nunha EDAR municipal. A configuración instalada no prototipo consistiu nun compartimento anóxico con biomasa suspendida, unha cámara aerobia na que coexisten biofilmes e biomasa suspendida e un compartimento de membrana (ultrafiltración de placa plana) onde tivo lugar a

separación da biomasa/efluente tratado. O sistema foi operado a un tempo de retención hidráulica (TRH) medio moi competitivo de tan só 6 h. As augas residuais caracterizáronse por unha relación moi variable da relación DQO_T/NT ao longo do día. A planta piloto eliminou de xeito estable sólidos e carbono orgánico nunha porcentaxe media de entre 90 e 100%, respectivamente. A eliminación de nitróxeno estivo limitada ao 49%. Aínda que o amonio nitrificouse completamente, a desnitrificación foi unha limitación na eliminación deste nutrinte. Un dos feitos que limitou a desnitrificación foi a escasa relación DBO_5/NT, especialmente nas etapas val do día onde se acadou este valor de tan só 2 $mgDBO_5/mgNT$, limitando a capacidade de desnitrificación. Ademais, existiu outra limitación: a da capacidade de bombeo da bomba de recirculación, desde a cámara de biofilmes ata a anóxica, que puido limitar tamén a eliminación de nitróxeno nas etapas nas que a relación DBO_5/NT era suficiente para desnitrificar. A nitrificación consome dúas veces a alcalinidade que desnitrificación libera. Como a nitrificación levouse a cabo completamente e a desnitrificación era limitada, este feito, xunto cunha baixa alcalinidade inherente do auga residual, conduciu a unha diminución do pH no efluente final por baixo de 4.5. Malia estas condicións extremas, para unha separación de licor mestura/efluente, os indicadores de rendemento da membrana foron sobresaíntes. A permeabilidade foi de 201 $L/(m^2 \cdot h \cdot bar)$ de media cando o fluxo se axustou a 20 $L/(m^2 \cdot h)$. O simulador Biowin predixo un comportamento similar ao do sistema real.

CAPÍTULO 4. Avaliación dun proceso combinado de UASB e BRM que trata augas residuais dunha industria de procesado de produtos do mar a diferentes temperaturas

As augas residuais altamente contaminadas xeradas durante os procesos produtivos, as facturas derivadas dos altos consumos enerxéticos e as severas demandas de auga son problemas comúns para moitas industrias de procesamento de alimentos.

O Capítulo 4 suxeriu unha combinación de reactores anaerobio UASB e BRM de dúas etapas en serie para tratar a auga contaminada dunha industria de manufactura de produtos mariños. Este capítulo propuxo un tratamento anaerobio, para a transformación da contaminación orgánica en biogás rico en

metano, empregable na mesma fábrica e diminuíndo as demandas externas de gas natural. Ademais, un BRM aeróbico acoprado en serie proporcionou as características de calidade de auga tratada para a súa posterior reutilización en aplicacións tales como lavado de vehículos, torres de refrixeración ou descargas de WC, diminuíndo a presión hídrica na zona. A temperatura na etapa anaerobia foi controlada (17-35 ºC) para evaluar o seu efecto no sistema. Éste foi capaz de eliminar de xeito estable DQO e sólidos nunha media de 94% e 100%, respectivamente. Lográronse rendementos óptimos a unha temperatura controlada de 30 ºC no reactor UASB con porcentaxes de metanización de ata o 90%. Ademais, o BRM foi capaz de contrarrestar as sobrecargas de DQO cando os rendementos na etapa anaerobia eran baixos. Iso levou ao cumprimento dos límites de descarga durante todo o período experimental. A presenza de compostos de amonio cuaternario (QAC) nas augas residuais procedentes de tarefas de esterilización e limpeza, inhibiu drasticamente os procesos biolóxicos nas etapas anaerobias UASB e BRM aerobia. Este feito indicou que a súa presenza debe ser xestionada con precisión nunha instalación a gran escala para evitar problemas de inhibición. O proceso de filtración (membrana de fibra oca) comportouse notablemente durante a primeira etapa da experimentación, con valores de permeabilidade tan altos como 356 L/(m^2·h·bar). Con todo, unha falla na automatización levou á operación da membrana por enriba das recomendacións do fabricante. Iso levou a unha diminución drástica na permeabilidade a valores tan baixos como 90 L/(m^2·h·bar).

CAPÍTULO 5. BRM como tratamento posterior de efluentes tratados anaeróbicamente.

O Capítulo 5 propuxo un sistema novidoso para paliar un problema moi coñecido cando se trata de sistemas de depuración anaeróbicos, especialmente cando se operan a baixas temperaturas, o metano disolto na corrente efluente. O metano considerouse un gas de efecto invernadoiro (GEI), cun potencial de quencemento 34 veces maior ca o CO_2. O contido de metano presente na corrente gas dun reactor anaeróbico é facilmente controlable e empregable. Con todo, se este composto está presente na fase líquida, eliminarase á atmosfera debido á turbulencia xerada na circulación da corrente líquida ou no reactor BRM de

adecuación do efluente instalado en serie. Por esta razón, o metano disolto debe eliminarse antes de que o efluente saia do sistema. A finais do século pasado, descubríronse microorganismos capaces de empregar este gas metano como doador de electróns sen custo, para desnitrificar. Neste capítulo, propónse un sistema para aproveitar este proceso microbiolóxico recentemente descuberto e aproveitar para corrixir a concentración de nitróxeno da auga residual mediante o uso do metano como doador de electróns na desnitrificación. Desta forma, pretendíase un obxectivo dobre, por unha banda, diminución da concentración de nitróxeno da auga tratada, con vistas a ampliar a súa aplicabilidade en reutilización, e para reducir os impactos ambientais relacionados co metano disolto. Utilizouse un reactor a escala bancada formado por un UASB (etapa anaeróbica) acoplado en serie a un BRM anóxico/aerobio (con membrana de fibra oca de ultrafiltración). O sistema foi alimentado con augas residuais lácteas de baixa concentración. En xeral, o sistema operouse a un TRH medio de 15 h. DQO e SST foron eliminados nun 95 e 100%, respectivamente. O metano disolto no efluente líquido do reactor UASB variou entre o 20-30% do metano total xerado, o post-tratamento MBR eliminou sistematicamente o 80% deste metano disolto. A nitrificación realizouse na cámara aeróbica do BRM e conseguiuse desnitrificar ata 15 mgNT/L. Aínda que os balances de masa indicaron que aínda se descoñecía unha fracción do metano eliminado, demostrouse que a desnitrificación con metano había ter lugar neste reactor. Atopáronse representantes microbiolóxicos, empregando a técnica FISH, e os ensaios en discontinuo deseñados para demostrar este feito. Con respecto aos indicadores de rendemento da membrana, alcanzáronse valores relativamente altos cunha permeabilidade que oscilaba entre 100 e 250 L/(m^2·h·bar).

CAPÍTULO 6. Descrición xeral da competencia entre os oxidantes de metano e amonio nunha contorna de escaseza de osíxeno.

No Capítulo 5, a principal complicación detectada no proceso global foi a falta de coñecemento nos complexos procesos microbiolóxicos involucrados na eliminación combinada de metano e nitróxeno. Este feito animou a continuar a investigación sobre este tema nun centro especializado nestas interaccións biolóxicas. Así, o Capítulo 6 centrouse en obter máis información sobre a

interacción dos ciclos de nitróxeno e metano, que podería ser aplicado nunha optimización adicional do sistema presentado no Capítulo 5. O Capítulo 6 levouse a cabo no Departamento de Microbioloxía da Radboud Universiteit Nijmegen (Países Baixos), recoñecida en todo o mundo pola súa investigación dirixida a coñecer os principais actores nos ciclos de nitróxeno e metano en contornas óxicas e anóxicas.

As primeiras tarefas realizadas neste capítulo baseáronse no enriquecemento das bacterias oxidantes de amonio (AOB) e as bacterias oxidantes de metano (MOB). No caso das AOB, executouse unha estratexia de enriquecemento en dous reactores continuos combinados en serie (Reactores A e B, 2 L cada un). Alternativamente, as MOB enriquecéronse mediante reactores discontinuos. No primeiro caso, fíxose funcionar un reactor en condicións axeitadas para promover a proliferación de AOB (escaseza de osíxeno, baixo tempo de retención de sólidos, TRS, instalando unha corrente de purga) pero a acumulación de nitrito non se detectou en ningún momento do período experimental. Como resultado das probas baseadas en PCR, as AOB estaban presentes sen ningunha dúbida, pero a ausencia de nitrito levou a pensar que outras poboacións estaban presentes no medio. Sospeitábase da presenza duns microorganismos capaces de oxidar de amonio completamente ata nitrato recientemente descubertos, commamox. Posteriormente, outras probas confirmaron a presenza destas poboacións. Como consecuencia do anterior, unha estratexia de enriquecemento descontinua substituíu aos experimentos continuos. Un cultivo puro de *Nitrosomonas europaea* foi seleccionado para ser enriquecido. Logo dalgunhas semanas de colleita, tiras de nitrito indicaron que as AOB estaban presentes en gran medida. Os cultivos descontinuos de *Methylomonas lenta* tiveron éxito xa no primeiro ensaio.

A segunda parte da investigación realizada no Capítulo 6 foi a procura de:

Nitrosomonas europaea:

I) A capacidade inhibitoria do metano (substrato MOB) na actividade AOB. Os experimentos indicaron que as AOB poderían ter actividade con metano de ata o

5% no espazo de cabeza. Concentracións máis altas inhibiron totalmente a capacidade oxidante das AOB.

II) Metano como substrato alternativo para as AOB. Previamente, reportouse que cando o amonio non estaba presente no medio, o metano podería empregarse como substrato para a actividade oxidante das AOB. Como resultado deste experimento, se a concentración de metano no espazo de cabeza representaba ata o 10%, as AOB poderían oxidar o metano, demostrando a capacidade de AOB para empregar o metano como substrato. Con todo, si a concentración de metano aumentaba ata o 20%, a capacidade oxidante só se observou nos primeiros 135 min de experimentación, o que indica que se detectou un efecto de envelenamento nos representantes de AOB. Polo tanto, o metano como substrato era só unha alternativa estable a baixas concentracións.

Methylomonas lenta:

III) Limiar inhibidor de amonio na actividade das MOB. Aínda que non se observou un efecto de envelenamento en ningunha das concentracións de amonio experimentadas (0-520 µM NH_4^+), observouse que unha maior concentración deste composto implicou unha menor capacidade de oxidación de metano. Alternativamente, cando o amonio estaba ausente e só o metano estaba presente no medio como substrato, non se detectou ningunha oxidación de amonio. Así, este feito, demostrou a incapacidade de MOB para empregar amonio como substrato, en contraste coa capacidade AOB de usar metano como substrato alternativo.

IV) O limiar inhibidor do nitrito na actividade das MOB. Atopouse que dependendo da súa concentración no medio, podía potenciar (99-250 µM NO_2^-) ou inhibir (1000 µM NO_2^-) o proceso oxidativo MOB.

Para rematar, realizáronse ensaios de seguimento do osíxeno biolóxico (BOM) para avaliar a concentración mínima de osíxeno disolto requirida para levar a cabo as actividades oxidantes tanto de *Nitrosomonas europaea* como de *Methylomonas lenta*. En ambos casos, atopouse que non se requiría unha concentración mínima de osíxeno disolto para iniciar a actividade biolóxica, o que

indica que non se necesitaba un limiar mínimo de osíxeno para realizar a oxidación biolóxica.

ACKNOWLEDGEMENTS/Agradecimientos

Toda esta aventura empezó oficialmente a principios de 2012, aunque ya previamente había ingresado en el Biogroup para desarrollar mi trabajo fin de máster. Me acuerdo en los primeros días, ante un Álvaro totalmente cohibido y tímido, las facilidades que tanto Edu como Tamara me habían brindado de cara a mi integración dentro del Grupo. A los meses, me "mudé al piso -1" donde, en fin, aparte de gestionar mis múltiples visitas a Lagares-Vigo y de analizar los resultados de las mismas, había tiempo para todo… Empezamos allí metidos Alberto, Santi Gen, Jeroni y yo… bautizando esa oficina como "*despacho alfa*" como no podía ser de otra manera… mucho macho allí metido… Evidentemente, no todo iba a ser trabajar… las cañas/escapadas y salidas nocturnas no se perdonaban… y todo ello con un toque muy cosmopolita, ¿verdad Ulises? el mexicano que no se perdía una… el mayor en edad y las lecciones de felicidad y de saber disfrutar de la vida que nos daba a todos, ¡wey! En este punto no quisiera dejar de nombrar a Teresita, la cual no desaparecerá de mi lista de agradecimientos ni siquiera en mi día a día actual, y Ali… dándole ese toque sevillano a todos los acontecimientos sociales que iban surgiendo. También en muchas ocasiones, teníamos el honor de compartir estos momentos con Tania, alias Palmeiro, otra de las que se han quedado en mi vida y ahí continúa, aunque se haya ido a Irlanda a investigar un poco más al norte de Europa. Mencionar también a Xitlalli y sus múltiples aventuras … que sé por seguro que se alegrará mucho de ver su nombre en estas líneas desde el cielo.

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Antes de cerrar el episodio "*despacho alfa*" quiero hacer mención especial a Alberto y también a Daga y a Leti… a quienes considero mis mentores en este mundo de la investigación. Sin vuestra aportación, seguro que ni este documento ni yo mismo seríamos lo mismo. Con un par de ellos tengo el placer de seguir trabajando a día de hoy y ¡que siga así por muchos años, porque claramente juntos somos mejores!

En este punto quiero hacer un aparte y acordarme de Natalia H., Adrián A. y Tomás. Con ellos he tenido la gran suerte de trabajar conjuntamente desde una perspectiva diferente. Chic@s, ¡ojalá hayáis aprendido de mí lo mismo que yo con vosotros! Ha sido una experiencia única y maravillosa trabajar con vosotros.

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Los aspectos familiares, se nutren también de la familia "que se elige", los amigazos. Sergio, Sabo y Adri; ocupando siempre un lugar muy especial. Al "Brandía Shore" [Samuel, Luisito, Roi, Tilve, Kike, Antonio, Penedo y los demás] que debemos ser un cuadro bastante curioso para quien nos ve desde fuera, "Nos vamos pronto para casa" [Víctor, Tati, Belén y Mario] grupo que se bautiza haciendo claramente uso de nuestro particular humor gallego, "Resistencia extrema" [Chimpi, María, Maciej] cuyo nombre hace referencia a los que quedamos en nuestra querida Compostela. Leti Salvado, siempre tan cerquita y con una visión tan mesurada del mundo. Olaia, agradeciéndole que siempre me pregunte por este documento, me anime a terminarlo y escuchando pacientemente mis chapas de *friki*. Peñaqua [Sonia, Isa, Manu, Patry, Guille, Alba], que aunque ahora muchos no compartamos el día a día, ha sido maravilloso que durante mis dos primeros años en Cetaqua hayáis estado ahí. Marta, que aunque nuestros caminos se hayan separado, ha sido soporte clave en mis primeros años de tesis.

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apoyo de un gran sistema educativo público dentro de una ciudad cuna universitaria y cultural también se puede hacer ciencia de calidad.

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Chapter 1

Introduction

Summary

This chapter aimed at overviewing the state of the art of membrane filtration in wastewater treatment and at contextualizing the motivation of the new developments with always a membrane installed in the flowchart. MBRs historical milestones, a technical background as long as the latest releases and hotspots are also included. Advantages and detected points to improve when comparing based on membrane approaches with traditional activated sludge treatments were identified. The main applications of membranes in wastewater treatment were: enlarging the wastewater treatment capacity occupying the same surface, turning the water quality of anaerobically treated effluents to reuse standards and acting as barriers for retaining specific slow growth microorganisms.

Urban and industrial full-scale facilities in which water reclamation was a crucial motivation were included to overview membranes' wastewater treatment worldwide. Available data of capital and operational expenses were also included as long as the energy consumption per cubic meter of treated water.

A 10-years bibliometric worldwide study, including papers and patents, was performed to forecast the scientific interest in the next years. Collected data encompass: overall scientific data, wastewater, wastewater with membranes and wastewater combined with greenhouse gas investigations.

1.1. General aspects of membrane filtration in wastewater treatment

Filtration membranes have been defined as porous materials that allow the selective pass of materials, depending on the compounds size. As a general rule of thumb, materials able to pass through the membrane should necessarily be smaller than the pore size of the membrane. Membrane pores have been typically classified by their pore size in: microfiltration (MF) 10^{-6}-10^{-7} m, ultrafiltration (UF) 10^{-7}-10^{-8} m or nanofiltration (NF) 10^{-8}-10^{-9} m membranes (Judd, 2011). For the cases of MF, UF, and NF separation, the driving force is exclusively governed by the application of pressure. Membrane filtration permits to address the separation towards the quality standards which allows a water reuse. Normally, the higher water quality required, the use of membranes with lower pore size and a higher energy consumption per volume of treated water is consumed. In the field of wastewater treatment, membranes are not exclusively applicable as a separation system to substitute a settler. Also, membranes could be used as a subsequent tertiary treatment of secondary treated wastewater, obtaining high-quality reclaimed water, facilitating water reuse.

The most common materials used to form membranes were either organic polymeric (PVDF, PES, PE, PP) or ceramic (Zr, Al, Ti oxides among others). Membranes are usually arranged as collections of elements housed in devices configured by the manufacturer, known as membrane modules. Their design was conceived to minimize the energy demands and to maximize the flux, defined as the amount of filtered water per membrane unit area and time.

A membrane bioreactor (MBR) can be defined as the combination of a membrane filtration process, as MF or UF, with a biological wastewater treatment step, as the activated sludge process. The separation between the mixed liquor and permeate is carried out by means of a membrane filtration process. The applicability of MBRs involved both municipal and industrial wastewaters. The first reference appeared in a conference held in Indiana (USA) in the late 1960s (Smith et al., 1969), where the industrial wastewater treatment using filtration membranes was presented for the very first time at laboratory scale. Later, an American company, Dorr-Oliver used membrane bioreactors for treating, at full-scale, the sewage produced in ship-boards

(Bemberis et al., 1971) by using a flat-sheet ultrafiltration membrane. In a first approach, the secondary settler, employed for the biomass/effluent separation in the CAS process was replaced by a side-stream flat-sheet membrane filtration system, in which permeate flows from the internal to the external part of the membranes (Figure 1. 1 a) with two outlet streams, permeate and retentate (Figure 1. 2 a). Membranes were located in a compartment exclusively designed to hold the physical separation of the mixed liquor/permeate. This approach has been known as external or side-stream MBR (s-MBR) (Figure 1. 2 a). Despite the attractive idea of coupling the biological treatment to a filtration membrane, the use of membranes was restricted to the treatment of industrial wastewater or those effluents generated on board in ships. Significant investment costs, high energy expenses (3-10 kWh/m^3 treated water) and the quick loss of performance of the physical devices of these first approaches (Judd, 2011) were the main reasons which counteracted a quicker growth of MBRs market at that time.

In the late 1980s, one of the greatest breakthroughs of the MBR technology occurred, with the development of the immersed MBR (i-MBR) (Figure 1. 2 b) (Yamamoto et al., 1989). In i-MBRs the membrane module is submerged directly in the bioreactor, diminishing the complexity of the tubing and pumping system associated to the previous s-MBRs. During the first 1990s, i-MBR technology with hollow fiber membranes was developed by the Canadian company Zenon Inc (now belonging to SUEZ) and the Japanese Mitsubishi Rayon. On the other hand, the Japanese Kubota developed at that time, flat-sheet membrane modules, significantly pushing up the market of MBRs. In these approaches, permeate flows from the external to the internal part of the membrane (Figure 1. 1 a/b). In these systems, coarse bubbles were applied to counteract the rapid loss of performance experimented in the membrane modules. With the development of i-MBRs, a drastic reduction of energy was obtained. In contrast, a decrease in the filtration capacity per surface area was detected, and higher membrane surface areas were needed for obtaining the same flow of permeate. Later, research efforts were focused on maximizing the treated flow, minimizing the process energy demands and optimizing operational parameters such as the mixed liquor suspended solids (MLSS), solid retention time (SRT) and hydraulic retention time (HRT) to enlarge the competitiveness of MBRs.
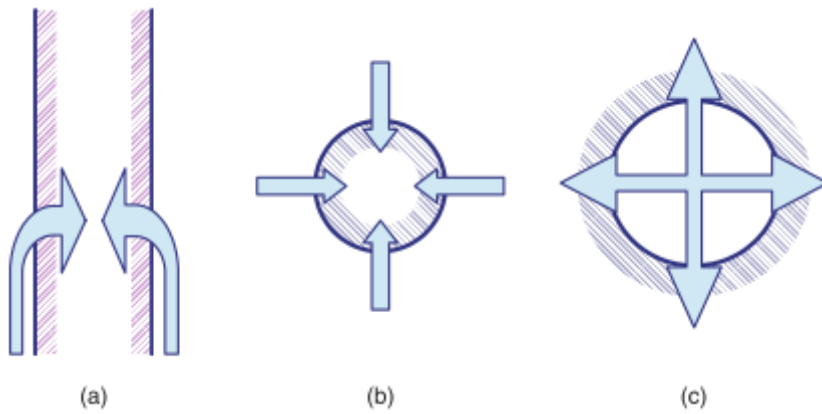
Figure 1. 1. Regimes of flow through a membrane: a) flat-sheet and b) hollow fiber membranes, out to inside flow (sidestream): the permeate flows from the external to the internal parts of the membrane; c) in to outside flow (dead end): the permeate appears in the external part of the membrane. Source: Judd (2006).
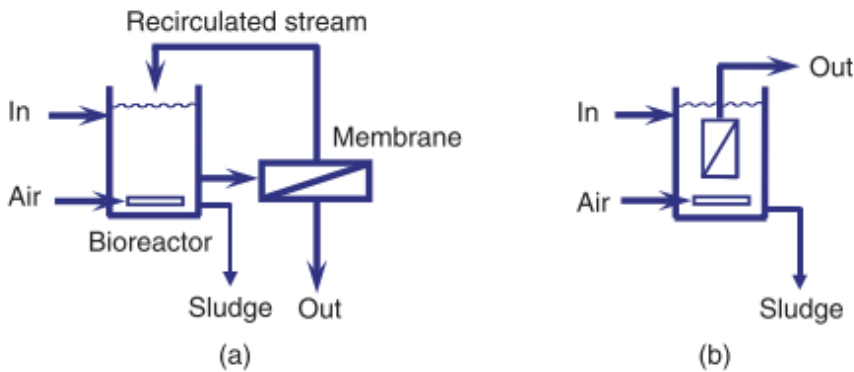

Figure 1. 2. Schematics of a) external or side-stream MBR (s-MBR); and b) immersed MBR (i-MBR). Source: Judd (2006).

From the first years since the birth of i-MBR technology, there were few membrane suppliers, the most known ones were Kubota (for flat sheet membrane modules), and Zenon or Mitsubishi Rayon (for hollow fiber modules) that installed most of i-MBR at that time. Moreover, this was a restricted market, dominated by these membrane manufacturers, which did not freely provide the access to the membranes to those

companies interested in their products, hampering the membranes market expansion. Nowadays, there are more than 40 membrane suppliers over the world (www.thembrsite.com; last access August 2018), implying an increase of both the competitiveness and the market size.

One of the typical inefficiencies found when operating MBR-WWTPs has been its operative capacity. MBRs in wastewater treatment plants (WWTPs) often operate at a regime significantly lower than their design flow. A smart solution was to increase the operating regime of the enabled modules up to a flow slightly lower than the maximum flux that could be achieved, below the level known as critical flux under which no or little fouling is observed. The remaining membrane trains, not required for treating the incoming flow, can be disabled. This could be accomplished considering the expected wastewater to be treated. By following this strategy, remarkable energy savings can be experimented (Krzeminski et al., 2016) since disabling modules only require maintenance cares in contraposition to the high energetic expenses if a continuous operation is conducted.

Another point to be optimized was the antifouling strategies; such as backwash, relaxation or air scouring and filterability indicators as off-site filtration tests or on-line transmembrane pressure (TMP) records. As filtration depended on many factors (Rodríguez-Hernández et al., 2014; Silva-Teira et al., 2018), the above-indicated parameters can be adapted to the filtration conditions. As e.g., backwash and/or relaxation periods can be adapted to the actual filtration status. Moreover, if favorable conditions appear, a decrease in the air flow or an intermittent aeration can be set up. On the other hand, reducing mixed liquor total suspended solids (MLTSS) can enhance an aeration save since microorganisms' basal oxygen demand, and its related air expense, can be minimized. In the past, MBRs were operated at an MLTSS 12-20 g/L; nowadays, this concentration was decreased down to 4-6 g/L. In previous decades, most of the currently available knowledge regarding MBR technology was still unknown. For this reason, many operational settings of MBR plants were drastically oversized, especially in terms of membrane surface area. In this sense, manufacturers' design recommendations were intentionally conservative to warranty the product to the client by minimizing the risks.

Newly appeared know-how and more experienced personnel in charge of the MBR-WWTP will definitely lead to an energetic demand diminution in the upcoming years. Recently, two of the main membrane suppliers Koch Membrane Systems and General Electric Water Division, the last recently purchased by SUEZ Environnement in 2017, launched more efficient membrane modules with views to a competitiveness increase of their products:

1) Puron PSH1800 (Koch). This optimized product has been able to rise the filtration capacity by 25% with a specific aeration demand decreased by 10% with respect to the competitors. This hollow fiber system based on the improvement of the membrane materials (Puron, 2011).

2) LEAPmbr ZeeWeed (GE SUEZ). Pulse aeration was developed to offer a reduction of 30% of the energy demand with a 15% increase in the productivity compared to the previously sold products (GE, 2011). Cycles in the membrane aeration for preventing membrane fouling instead of the former continuous aeration was the main driver in the costs reduction.

Based on some of the previously described strategies, Tao et al., (2010) observed a specific energy demand drops from 1.3 down to 0.4 kWh/m^3 in a long-term 6 years study at pilot scale, demonstrating that there is still a significant margin in the optimization of the currently in use full-scale MBR-WWTP. Apart from energy consumption, MBRs can improve their competitiveness if an increase in the wastewater treatment capacity, per volume of the reactor, is gained by maintaining the energetic demands.

In the last two decades, the concept of MBRs has been widened. As an example, a combination of anaerobic methanogenic treatments and membrane filtration units appeared and was named anaerobic MBRs (AnMBR). This technology combines the benefits of anaerobic processes (methane-rich biogas is obtained and high organic loading rates) with membrane filtration units (lower footprint and higher quality of the effluent). These systems could be set up following either i-MBR or s-MBR configurations (Figure 1. 2) with dead-end or side-stream layouts, respectively. AnMBRs have been typically installed for treating industrial wastewaters.

From 2003 onwards, the concept of hybrid-MBR was also developed. It has been defined as a combination of flocculent and biofilm (biomass attached to carriers) biomass in order to improve the biological activity in the MBR system. The goal of this new alternative was not only enlarging the capacity of the system but promoting specific microbes onto the biofilm was also aimed. Its applicability included both municipal and industrial wastewaters. It allowed decreasing the HRT of the system, making it compacter and increasing the treatment capacity per unit of occupied area. Biofilm presence also improved the membrane filtration performance, since protozoa growth, able to remove colloidal particles for the membrane filtration process, were promoted (Buntner et al., 2013). By adding the small carriers an equivalent increase of 1.5-2.0 g/L of activated sludge was attained (Metcalf&Eddy et al., 2014) positively impacting on the treatment capacity of the system. SUEZ Meteor® (biofilm biomass exclusively) and Meteor IFAS® (flocculent and biofilm biomasses) are commercial references based on this concept. The patent "Hybrid biological reactor of membranes for the treatment of industrial and municipal wastewaters" (EP 1 484 287 B1), belonging to the University of Santiago de Compostela, describes a hybrid-MBR technology to treat both industrial and municipal wastewaters, and probably was one of the first hybrid technologies developed.

MBRs as post-treatment of anaerobic treatments also appeared. Well-known issues when dealing with anaerobic reactors were their incapacity of reducing nutrients of the wastewater and the high presence of solids in the effluent. Newly appeared MBR post-treatments were able to polish anaerobic reactors' effluents. The patent "Three-stage biological reactor of membranes (methanogenic, aerobic and filtration) for the wastewater treatment" (ES 2 385 002 B2) describes a system in which the organic carbon was majorly transformed into methane-rich biogas in an anaerobic stage. Then, a two compartments aerobic MBR was set up. In the first, plastic carriers were colonized with specialized microorganisms to degrade the remaining organic carbon. In the second, an ultrafiltration stage was held to obtain a high-quality effluent (Buntner et al., 2013). Other patented system features an anaerobic methanogenic reactor, in which the major fraction of the organic carbon is methanized, and a two stages MBR in which both the remaining organic carbon and nitrogen are removed (ES 2 401 445 B2 5). In this latter stage, newly discovered microorganisms able to denitrify by employing the dissolved methane coming from the anaerobically treated effluent as a

carbon source (Sánchez et al., 2016; Silva-Teira et al., 2017) are hosted. In this way, diffuse emissions related to the methane dissolved in the anaerobic treated wastewater could be counteracted and nitrogen concentration in the effluent can be corrected, widening the applicability towards a water reuse.

1.1.1.   Drivers for the MBR implementation

Drivers for the MBR full-scale expansion (Judd, 2011; Krzeminski et al., 2016):

a) More stringent legal discharge limits in sensitive areas due to an increasing awareness of both society and policymakers,

b) a water reuse can be obtained due to the outstanding quality of the reclaimed water, conventional pollutants and parameters of water quality as pathogens as long as the total retention of solids,

c) significantly lower land demands for its implementation in comparison to traditional conventional activated sludge (CAS) systems,

d) an excellent option when an upgrade/retrofit of an already constructed WWTP is needed.

1.1.2.   Comparison of MBR with CAS systems

A conventional activated sludge (CAS) system is the most expanded technology worldwide to biologically treat the wastewater. It includes an aeration tank, which is used for the biological degradation of the pollutants, and a second clarifier, sedimentation tank, where the sludge is separated from the treated water.

When comparing a traditional CAS and an MBR (defined in the "general aspects" section), some well-known drawbacks were detected which slew down MBRs global expansion in the new built WWTP with regard to CAS, especially is a water reuse quality of reclaimed water is not a purpose:

a) Economic related issues:

1) Capital expenses (CAPEX), since the infrastructure, control and instrumentation have been normally more complex than CAS requirements. Membranes are typically expensive elements.

2) In the past, it was claimed that operational expenses (OPEX) in MBR have been normally higher than those observed with traditional CAS systems. Energy expenses for fouling mitigation (definition below) in aerobic treatments or the applied pressure in anaerobic reactors have typically accounted as one of the main expenses of the operational costs.

b) Membrane fouling: defined as the deposition of solid particulates onto the membrane surface, blocking the pores and diminishing the filtration capacity. This phenomenon usually promoted a decline in the membrane performance indicators (permeability and transmembrane pressure, TMP) and its lifespan (Iorhemen et al., 2016), averaged in 10 years;

c) Traditionally, much lower cumulated knowledge in comparison with CAS systems, which makes decision-makers reluctant to install the MBRs in new facilities.

Fortunately, in the last years, many efforts aimed at overcoming these abovementioned weak points were executed. As a result, much more knowledge was collected in fouling counteraction and increased performances were observed. Accordingly, more competitive values of OPEX/CAPEX in comparison to CAS systems were lately observed (Iglesias et al., 2017), breaking the barriers towards its full-scale expansion.

1.2. Technical aspects

1.2.1. Operational parameters

Flux (J) is defined as the flow per surface area unit of the membrane, Equation 2. A common practice when dealing with MBRs has been their operation under a certain flux.

Transmembrane pressure (TMP) is the difference of pressure between the mixed liquor and the permeate, should be applied in order to maintain the flux stable in the filtration process. Equation 1 indicates its calculation. TMP is associated with pumping energy consumption,

The permeability of a membrane is the relationship between the flux applied divided by the transmembrane pressure observed. This parameter indicates the efficiency of the filtration and it was defined in Equation 3. Hence, the higher permeability implied a lower energetic demand to maintain the set flux and an increased performance of the filtration process.

$$TMP_A = P_{IN} - P_{OUT}$$
Equation 1.

$$J = \frac{F}{A}$$
Equation 2.

$$Permeability = \frac{J}{TMP}$$
Equation 3.

where:
J is the flux [L/(m^2·h)], F the flow [L/h] and A the filtration surface [m^2]. TMP is the transmembrane pressure [bar]. Consequently, the common units to express the permeability are [L/(m^2·h·bar)].

1.2.2.   Fouling, clogging, and strategies for their minimization

Fouling is the effect of the convection and the accumulation and/or adsorption of foulants onto the membrane internal/external surface, decreasing the membrane performance (Drews, 2010; Judd, 2011; Le-Clech et al., 2006). Fouling leads to an increase in the energy demands of MBRs.

Two interesting characterizations were established to further understand this phenomenon: a) fouling typologies and b) main foulants:

a) A brief classification was published to define the type of fouling as a comparison to the optimal performance of the membrane. According to Drews (2010), fouling can be:

1) Reversible. It occurs due to an external deposition of materials onto the membrane surface, leading to a TMP increase. TMP can be decreased by means of physical cleaning methods such as backwashing or relaxation. This fact can be counteracted by applying an air sparging stream onto the membrane surface.

2) Irreversible. It has been defined as the fouling only removable by chemical cleanings to recover previous TMP levels. As common chemicals employed are sodium hypochlorite (to oxidize organic carbon depositions) and citric acid to remove the inorganic fractions. Intensive chemical cleans have been typical solutions to recover the previous high performance states of the membrane.

3) Irrecoverable. Inherent in the membrane usage and its lifespan. There are neither chemical nor physical strategies to recover the efficiency.

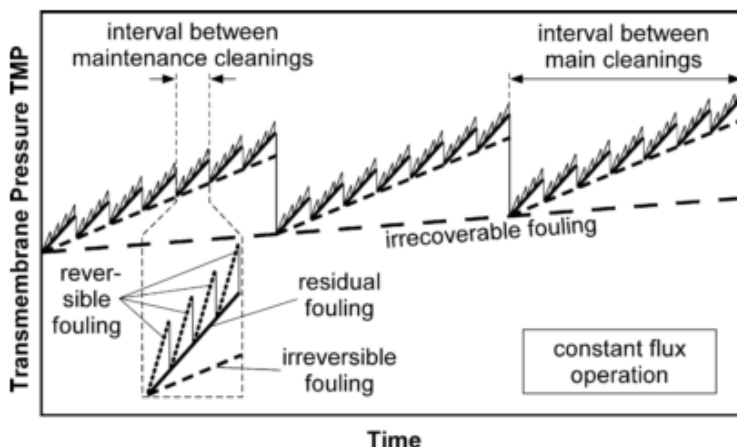Figure 1. 3 graphically showed the above-explained fouling mechanisms.

Figure 1. 3. Graphic representation of the types of membrane fouling and its impact on the transmembrane pressure (TMP). Source: Drews (2010).

b) Fouling is a very complex phenomenon involving factors such as membrane design parameters, hydrolyzed products of the reactor or sludge deposition, among others. In the past, there were large research efforts to correlate fouling with some chemical indicators, many times with little success. Listed below are the most common substances and indicators which have been typically used as measurable substances to describe the membrane fouling:

1) Soluble microbial products (SMP). Colloidal and soluble biopolymers, mostly carbohydrates and proteins (Le-Clech et al., 2006).

2) Biopolymer clusters (BPC). Fraction of colloids retained by the membrane, measured as the difference between total organic carbon (TOC) between the mixed liquor and permeate (Sánchez et al., 2013).

3) Extracellular polymeric substances (EPS) (Gao et al., 2011) or transparent exopolymer substances (TEP) (Drews, 2010) as other common parameters.

Although lots of additional knowledge was gained in the last decades, fouling has been still considered a phenomenon enormously difficult to be described. Many factors are involved in fouling as the sludge biological status, its rheology as long as other parameters as the pH, temperature, dissolved oxygen (DO); amongst many others (Le-Clech et al., 2006).

Based on the above-described information, different physical or chemical strategies were developed to diminish this undesirable fact. Most common strategies, applied for i-MBRs are described below:

a) Physical:

1) Aeration. A scouring effect on the cake layer formed when the permeate is sucked out from the mixed liquor has been aimed when applying an intense aeration stream on the membrane surface. The specific aeration demand based on membrane surface has been defined as the flow of air which should be applied to the membrane to maintain the optimum filtration capacity. This parameter is normally indicated by the manufacturer in terms of $m^3/(m^2 \cdot h)$, referred to the air flow and the membrane surface.

2) Backwashing/backflushing. Procedure in which the flow direction of water is reversed periodically through the membrane. A reduction of the cake layer is expected when this strategy is applied. This strategy is normally conducted in each cycle in a period of time much shorter than the standard filtration period.

3) Relaxation. Stablishing a pause in each period of time. In this fraction of the time, neither filtering nor backwashing is performed through the membrane.

Either backwashing or relaxation or both should be implemented only in the case in which these strategies are recommended by the manufacturer. These two approaches are generally compatible with the aeration which has been traditionally set up the whole time when the membrane is in contact with the mixed liquor. Fortunately, last operational recommendations indicated that aeration in cycles can be applied, entailing important energy savings, without a loss of performance in the filtration process. Aeration/non-aeration periods should be indicated by the membrane manufacturer. These three items have been addressed to counteract the above-mentioned "reversible fouling", Figure 1. 3.

The type of wastewater treatment and the recommendations of the membrane supplier determine the strategy for preventing the membrane fouling. For instance, air sparging is not permitted if the membrane is submerged in an AnMBR and special robust membranes have to be installed sparged with other gas, typically the biogas obtained in the anaerobic reactor. Conclusively, researchers have to study the system as a whole in order to meet the requirements not exclusively of the membranes also of the involved biological system present.

b) Chemical:

1) Chemically enhanced backwashing. It consisted of an external chemical addition in a frequency indicated by some manufacturers. Added concentrations should be relatively low to prevent damages to the membrane, normally below 100 mgNaOCl/L.

2) Maintenance cleaning. This protocol helps to reduce the frequency of the intensive cleanings. It employs an intermediate concentration of chemicals (100-600 mgNaOCl/L) between the "chemically enhanced backwashing" and the "intensive cleaning". When applied, its periodicity is about once a week or other, depending on the manufacturer instructions.

3) Intensive cleaning. Mainly focusing on the "irreversible fouling", Figure 1. 3 relatively high concentrations of chemicals are required. Only in this case, the continuous operation has to be stopped for performing this task. As this treatment has been considered exhaustive since the high chemicals concentrations added (1,000-2,000 mgNaOCl/L), it is normally carried out once/twice a year.

Special care should be taken with the dosage of chemicals. Literature, technical reports, and manufacturer's suggestions have to be accurately considered since an excessive chemicals addition can damage the microbial community and a too low concentration can lead to not to obtain the desired effect.

Another phenomenon able to diminish the membrane filtration capacity has been known as clogging. It was defined as the agglomeration of coarse solids that are accumulated at the entrance or within the membrane channels. This event partially blocks the filtration capacity (Drews, 2010) of the overall device. Drastic methods such as manual washings, chemical cleanings coupled to jets of water and manual agitation have been typical strategies for restoring acceptable permeability values (Zsirai et al., 2012). Those can be applied individually or as a combination of them. Normally, these practices involved the undesirable necessity of stopping the membrane filtration.

As a membrane-based system has been normally more complex than conventional systems, normally the presence of qualified and experienced personnel is crucial in these facilities.

1.3.　MBR references for wastewater treatment

1.3.1.　Municipal wastewater

High rate population growth and its concentration in big centers have been two of the main characteristics of the population trend in the last decades and future forecasts are addressed accordingly, as reported by United Nations (United Nations, Department of Economic and Social Affairs, 2017). This paradigm entails a double necessity: 1) an incremental demand of high-quality water for meeting population necessities itself and its related human activities and 2) a positive progression of compact wastewater treatments for cleaning the water without putting in risk the conurbation surrounding ecosystems, also attending the scarce of land availability in those areas.

MBR-based technologies can fulfill this double aim since the treated water by means of a membrane typically provided a high-quality output, reusable in many applications such as irrigation and/or street cleaning, among others, also depending on the local legal constraints. Although treatment costs are still slightly higher when a membrane is present (filtration driving force and antifouling strategies are energy demanding processes) in comparison to CAS systems, when a tertiary treatment is set up with views to a water reuse, CAS + tertiary and MBR are involved in a fair competition, this fact will be further reported when current costs are studied in this Chapter 1. Likewise, in areas where the hydric stress has been a traditional and present reality, research and development (R+D) efforts have been intensively addressed to MBRs as one of the main cores of research. Table 1. 1 reflects the largest infrastructures worldwide based on MBRs for the wastewater treatment ordered by their treatment capacity.

Table 1. 1. Largest worldwide municipal MBR-WWTP installed/planned.

WWTP	Peak daily flow (m³/d)	Year	Location/Country	MBR Company
Henriksdal	864,000	2019	Stockholm/Sweden	GE WPT
Seine Aval	357,000	2016	Acheres/France	GE WPT
Canton	333,000	2015	Ohio/USA	Ovivo
Euclid	250,000	2020	Ohio/USA	GE WPT
Yunnan	250,000	2013	Kunming/China	OW
Shunyi	234,000	2016	Beijing/China	GE WPT
Macau	210,000	2017	Macau/China	GE WPT

Two WWTP has been selected for a further description as reference plants in based on membranes wastewater treatment infrastructures. The city-state of Singapore is an example. Although the rainwater is abundant, natural aquifers and groundwater reservoirs are inexistent in this island. Last predictions indicated that the continuous rise of water demands cannot be fulfilled only with these rainwater incomes. Hence, researchers have been encouraged to explore new alternatives in water catchments. Newly constructed municipal treatment plants, known as *NEWater* facilities have been recognized as worldwide well-known examples, Figure 1. 4. Based on advanced membrane technologies; as ultra (UF), micro-filtration (MF) and reverse osmosis (RO); these infrastructures have been able to change the perspective from facilities in which the wastewater was cleaned towards alternative sources of high-quality water. As a result, the reclaimed water through this via is currently providing more than 30% of Singapore populations' water needs, with views to achieve up to 50% of water demands in 2060. To highlight is the treated water quality, higher than the World Health Organization's drinking water recommendations, allowing these effluents to be reused even as drinking water for the Singaporeans (Public Utilities Board Singapore, 2012). It implied that these days, 100% of the water treated in *NEWater* WWTP is reused in this country. The next challenge was to make this regenerated water more affordable than the present rates and the specialized R+D staff is currently working on these items. These *NEWater* facilities were the result of either constructing new plants or upgrading the existing ones, where biological treatments, sedimentation tanks, and subsequent MF/UF treatments were merged in one single basin, an MBR, entailing an

enormous space saving. Figure 1. 4 depicts the flowchart of the CAS transformation to MBR-based WWTP.
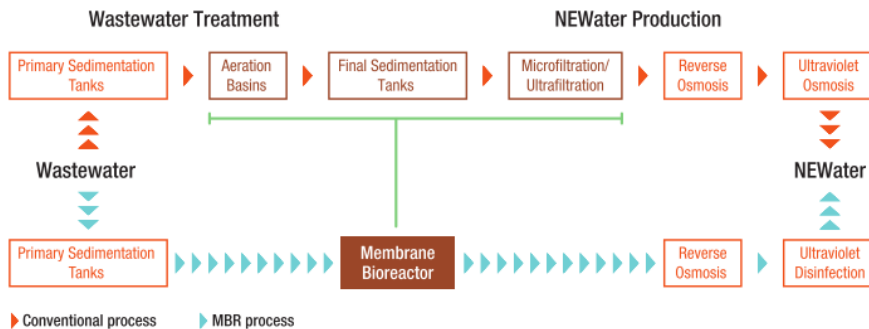

Figure 1. 4. Comparative scheme approaches when a conventional CAS and an MBR are setups in Singaporeans WWTP. Source: Public Utilities Board Singapore (2012).

Besides, the capacity of treatment of the MBR systems can multiply by three times (Krzeminski et al., 2016) conventional systems, entailing significant land savings in comparison to those wastewater treatment plants (WWTP) based on traditional CAS approaches. Hence, MBR new references are being set up in many WWTP in which an enlargement of the capacity is needed. Cities which experimented a rapid growth in the last years, increasing both wastewater treatment demands and water necessities, places where the hydric stress is a current trouble or discharge points which are protected ecosystems or sensitive areas (Iglesias-Obelleiro et al., 2012) have been market niches where MBR-based systems intensively enlarged. The largest worldwide MBR municipal WWTP is an upgrade of the Henriksdal WWTP (Figure 1. 5) placed in Stockholm-Sweden (increased capacity up to 864,000 m^3/d) meeting two of the above-mentioned conditions, scarcity of land and strict discharge limits. Among the list of technologies studied, decision-makers have chosen MBR since Stockholm is one of the European population quickest growing cities (1.5% per year) and the Swedish government is really committed to the environment. Effluent characteristics should accomplish very restrictive limits before entering the receptive water body (SUEZ, 2016). The start-up of this facility is planned for the beginning of 2019.
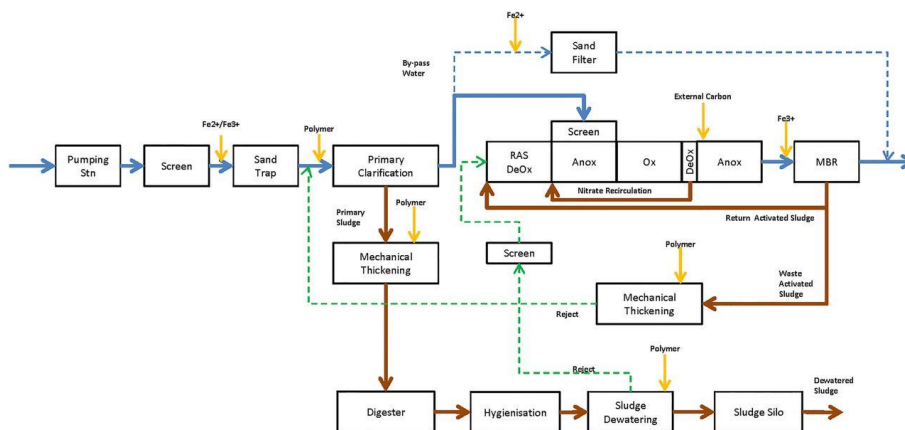
Figure 1. 5. Flowchart of the future Henriksdal WWTP (Stockholm-Sweden). In blue is the waterline and in brown is the sludge line. Source: SUEZ.

1.3.2.   Industrial wastewater

Industrial wastewaters are typically largely polluted than municipal outputs. Although the produced flows have been normally much lower than the municipal ones, their massively polluted characteristics can deeply damage the surrounding ecosystems (European Commission, 2006). For preventing this fact, an adequate wastewater treatment has to be necessarily applied. Legal effluent limits strongly depended on the discharge place and the awareness of society and policy-makers about environmental aspects. If the industry has to emit to a municipal sewer collector, those values are normally less restrictive than those needed if the discharge enters directly to a natural body, such as the sea or a river.

Chemical industries (M$ 605,000 in EU and M$ 581,000 in US, in 2014), food & beverage (M$ 696,000 in EU and M$ 415,000 in US) and pharmaceuticals (M$ 312,000 in EU and M$187,000 in US) have been included among the sectors in which the economic activity has been recognized as the most important ones in both, Europe and the US (Bluetech Research, 2014). Unfortunately, their productive processes triggered strong hydric demands from the surrounding water sources. Moreover, the wastewater treatment usually implies a significant economic share on the final price of the manufactured good, especially in sectors in which the added value is relatively low, such as the food & beverage industries.

In the above-stated industrial sectors, there are two costly items related to the water to be taken into account in the final price of the produced good: 1) potable water and 2) the wastewater treatment. The first typically entails a purchase tax. In the second, the treatment of the wastewater accounted for two expenses: those costs directly related to the wastewater treatment itself and the dumpling taxes associated with the water discharges to the public domain. These two items encouraged the companies' directives to assess wastewater treatment approaches for reducing both energetic wastewater expenses and the investigation of water reuse strategies to increase their competitiveness. For food & beverage, in terms of water reuse, it is not foreseeable the employment of reclaimed water directly in the productive processes in the next decades if any post-treatment is set up after the MBR (Bluetech Research, 2014). Fortunately, it can be currently employed in auxiliary activities. Washing trucks, floors or tanks, WC flushing, irrigation applications, cooling/heating processes are purposes in which the required water quality has been fulfilled by means of an MBR treatment. Other applications, in which the direct contact to edible goods, are not legally allowed.

The paradigm of the wastewater treatment is changing. In the last years, the polluted water has been viewed as a cradle of resources, laying aside the perspective of only a waste to be managed. This new perspective directly changed the perspective aiming at obtaining valuable products such as feedstocks (VFA, PHA, PLA, methanol, ethanol …) or bioenergy (biogas), employable in the same or other factories. Different methanogenic technologies for obtaining bioenergy from the wastewater are already on the market, with several references belonging to the main environmental technology companies worldwide such as SUEZ, Veolia or Paques. In this sense, the additional use of filtration membranes warranted a high quality of the treated effluent which can be reused, among others, directly in the facilities. Below are summarized a clear example of how to take profit of the resources contained in the wastewater and how to diminish the water demands from the natural bodies.

Unilever Marmite® and Brovril® production facility, UK

The Unilever factory produces two types of commercial sauces, Marmite and Bovril, commonly employed for cooking meat in houses and restaurants. The productive process generates a high polluted wastewater which is directly managed in a WWTP located in the same factory.

Figure 1. 6 includes a flowchart of the wastewater treatment process. Basically, this system is a combination of an anaerobic treatment and an MBR to provide to the treated water a high quality with views to a water reuse.

Figure 1. 6. Simplified flowchart of the wastewater treatment in a Unilever factory (UK). Source: Bluetech reports (2014).

As an influent to the anaerobic treatment two streams are mixed, the wastewater from the productive process and the centrate of the sludge dewatering. This mixed carbon-rich stream is fed to an anaerobic reactor, featured in an Expanded Granular System Biobed (EGSB®) technology of Biothane Systems (Veolia). This compact approach consisted of a high rate anaerobic expanded granular system able to treat high organic loading rates (OLR), up to 30 kgCOD/m^3·d. The organic carbon degradation outputs a methane-rich biogas (75% CH_4) directly valuable as energy. The reported benefit is estimated in meeting over 10-15% of the overall energetic demands of the factory. Coupled in series to the anaerobic reactor an ultrafiltration MBR was installed. The MBR's effluent is split into two streams. 1) The major share was directly driven to an RO module which allowed to reuse the 45% of the incoming flow to the industrial

wastewater treatment plant (WWTP). 2) The remaining 55% was the sum of both RO rejection and the non-reusable water coming from the MBR. This stream was discharged to the municipal sewer and fulfilled the legal requirements.

1.4. MBR market evolution and perspectives

1.4.1. Municipal field

Current facilities retrofit/upgrade, space limitations and stricter legislation with regards to discharge or reuse limits have been the main drivers of the MBR's worldwide spread in the urban scenario.

By the end of 2008, 800 commercial MBR were in use. Membrane prices significantly decreased over the last 15 years leading to a rise of the available references, especially in medium size facilities (10,000-100,000 PE.). The growing interest for the MBR as long as the increasing number of references led to a much higher expertise gained about their operation and design.

The current evolution of the MBR market has been undoubtedly growing in the last years. Already published forecasts agree in the fact of the growth will continue in the next future. Nevertheless, to date, this expansion is not been as quick as previously expected. In 2008, an estimated compound annual growth rate (CAGR) of 22.4% from 2008 to 2018 was foreseen (http://www.waterworld.com; last access July 2018). Updated studies were more conservative reducing the CAGR down to 12.8% in the period 2014-2019 (http://www.prweb.com, last access July 2018). These corrected predictions were caused by the MBR OPEX in comparison to the CAS systems. MBR OPEX is still higher than CAS especially if water reuse is not a purpose. On the other hand, MBRs are strongly competitive with CAS if water reclamation is desired. In many cases, as when land availability is an issue, MBR undoubtedly beat CAS.

1.4.2. Industrial field

As stated above, hydric pressure nearby the location of the factories has been a common concern in the most important industrial sectors worldwide. Aware of this situation and aimed at improving their public image, huge companies such as Coca-

Cola®, among others, designed plans to reduce the water consumption ratio from the surrounding areas. MBR-based technologies produce high-quality effluents with low organic pollutant concentration and microbial indicators, which allows a feasible water reuse. If the factory produces food or beverage outputs, the restrictive legal requirements limited the uses of the reclaimed water to purposes in which non-potable water was demanded. Water for cooling towers, vehicles washing or WC flushing are applications in which MBR effluents have been an alternative option.

In the next years, the use of MBR-based technologies will foreseeably grow. Policymakers in Europe are tending to increase the drinking water taxes. In this way, an economic pressure increase to diminish the water catchment by the industry has been targeted. These measures encouraged the establishment of MBRs.

New MBR-based technologies offered much more energetic efficiencies in comparison to the former traditional systems. Moreover, water reclamation remarkably decreases water catchment from the natural bodies. These two characteristics directly impacted in the expenses related to water, drinking water purchase expenses and discharge fees. Moreover, if biogas is obtained in the treatment a reduction in the gas invoice was definitely observed. The combination of these facts can decrease the investments performance rates to paybacks as low as 3-4 years, strongly encouraging to the industries' directives to install MBR technologies. No predictive data has been found due to the difficulty of obtaining reliable data.

1.5. Reclaimed water destination and economic/energetic expenditures in municipal MBR

MBR is one of the best available technologies (BAT) for water reuse (Lorenzo and Vega, 2010). By treating the wastewater with an MBR-based technology, such a high-quality effluent is obtained that the permeate can be used for many purposes. In this section, the latest available data regarding both capital and operational expenditures have been collected.

In Spain, reclaimed water has been typically addressed to aquifer recharge (43%), agriculture (31%), irrigation of golf fields (23%) and urban purposes (3%) (Iglesias et al., 2017).
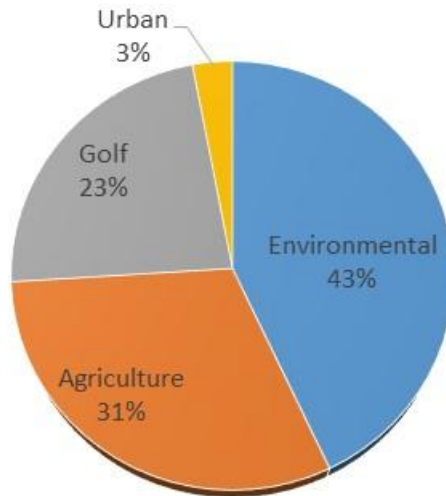
Figure 1. 7. Reused water purposes in Spain in the municipal market. Source: Iglesias et al. (2017).

Micro, ultra-filtration, and reverse osmosis have been the membrane processes used to fulfill the water quality standards of the Spanish water reuse decree (RD 1620/2007). Stricter values of the reclaimed water will be a reality in the next few months. The Joint Research Center (JRC), an advisor authority for the European Commission, has recently recommended new stricter values of the reclaimed water for the water reuse in agriculture (Alcalde-Sanz, 2017) to the European Commission. These recommendations were proposed by the European Commission in May 2018 for an official regulation to the both European Parliament and Council.

As an example, Table 1. 2 includes a comparison between RD 1620/2007 and the JRC recommendations for the strictest Class A classification, for agricultural irrigation. Class A contains food crops irrigation, including root crops consumed raw and cases in which the direct contact between reclaimed water itself and the edible portion of the god (Alcalde-Sanz, 2017) exists.

Table 1. 2. Comparative between the Spanish RD 1620/2007 and the recommendations of JRC to the European Commision 2017 for water reuse for agricultural purposes (Class A).

	RD 1620/2007	JRC 2017
Esterichia Coli (cfu/100 mL)	≤ 100	≤ 10 (or below detection limit)
Legionella spp. (cfu/L)	≤ 1,000	≤ 1000 (when risk of aerosolization)
Helminth eggs (egg/L)	-	≤ 1 (when irrigation of pastures or fodder for livestock)
TSS (mg/L)	≤ 20	≤ 10
Turbidity (NTU)	≤ 10	≤ 5
BOD$_5$ (mg/L)	-	≤ 10

Iglesias et al., (2017) have studied real data collected from 11 different full-scale facilities in Spain treating and reclaiming municipal water. In terms of operational expenditures (OPEX), it was observed a wide spectrum of values mainly due to two reasons: 1) the influent characteristics and 2) the operational flow. In Spain, the treatment capacity set in the MBR-based WWTP has been normally remarkably lower than the design parameters. This fact led to the loss of the economy of scale factor since the equipment always demanded minimum energetic expenses to run. The aforementioned publication has recently collected OPEX varying from 0.215 to 2.590 €/m^3 of treated water. The direct average value was 0.91 €/m^3 (of treated water). If the two highest data were discarded (due to the low performance in the operation of those facilities) the average cost would be 0.58 €/m^3 referred to the treated water).

22 full-scale Spanish CAS WWTP were studied including water and sludge lines management costs. OPEX ranged between 0.04 and 0.34 €/m^3 (of treated water), averaging 0.14 ± 0.07 €/m^3 (Lorenzo-Toja et al., 2016). Despite this lower OPEX in comparison to MBR-based systems, the referred system did not offer the water reuse benefit. A tertiary treatment should be further applied to meet the legislated quality standards needed for the water reuse. Typically, physical and chemical treatments, such as sand filters/UV lamps and disinfection (chlorination/ ozonization), respectively,

have been installed as tertiary treatments if the water reclamation is aimed. Those treatments have entailed additional treatment costs of 0.06-0.09 €/m^3 (of treated water) (Iglesias et al., 2010) to add to the inherent wastewater costs in a CAS system, based on real data compilation of Spanish WWTP real management costs. The overall treatment fee increased up to 0.21 €/m^3 (of treated water) if water reclamation was desired.

In brief, despite the traditional idea of MBR treatments have been more costly than CAS, two considerations should be taken into account when making a comparison: water reclamation and WWTP efficiency. In the scenario of a water reuse, when a tertiary treatment is needed, if the MBR facility is efficiently managed, an OPEX comparison between CAS + tertiary and MBR have become competitive in the last years. Previous studies, focusing on real Spanish OPEX compilations in MBR and CAS based WWTP, respectively, (Iglesias et al., 2017; Lorenzo-Toja et al., 2015), put aside the ancient assertion of membranes are always more expensive than CAS systems, if water reclamation is required.

Regarding the consumed energy in the 11 WWTP's included in Iglesias et al., (2017), a common particularity appeared in all of them. The energy consumed by the MBR in comparison to the overall energy expenses represented 28 to 34% (0.19-1.71 kWh/m^3) of the overall energetic requirements of those WWTP (0.55-3.27 kWh/m^3). Other references placed the energy consumption in the same order, ranging from 0.6 to 2.3 kWh/m^3 (Krzeminski et al., 2016), pointing that an optimal operation can diminish this rate down to 0.4 kWh/m^3. For this reason, MBR's optimization will definitely lead to an increase in the competitiveness of MBR respect to traditional CAS systems.

In terms of capital expenditures (CAPEX), CAS + tertiary treatment real costs ranged from 730 to 850 €/m^3 (of treated water). The MBR cost for similar characteristics varied from 700 to 960 €/m^3 (of treated water) (Iglesias et al., 2017). Both CAS + tertiary and MBR costs were not decisive from a CAPEX point of view. This study reflected the expenses related to the infrastructure, excluding the land purchase.

There are already available estimations of both CAPEX and OPEX in the literature. Cost analyses were performed by itemizing each component of a typical scheme. (Lo et al., 2015) accurately studied three plants of different sizes 100, 500 and 2,500 m^3/d

to find out a mathematical correlation to estimate both CAPEX and OPEX as a function of the treated flow. The obtained trend was in good agreement with other larger-scale studies (Young et al., 2012).

1.6.   Bibliometric analysis

A study of publications, excluding patents, found in the database Scopus (www.scopus.com; Elsevier B.V.) was conducted to evaluate the research trends observed in the last years in the field of membrane bioreactors for treating wastewater. Thus, the words: "membrane bioreactor; wastewater" were inserted in the website's field "keywords" and it was compared to other searches as: "wastewater", "greenhouse gas, wastewater" and the overall scientific output published in the same period of time, according to Nature. This study includes all documents of the database in the last 10 years, period 2008-2018. Data have been updated in June 2018.

Figure 1. 8 reflects the documents normalized to those published at the beginning of this analysis in 2008 with the inserted keywords above-indicated in Scopus.
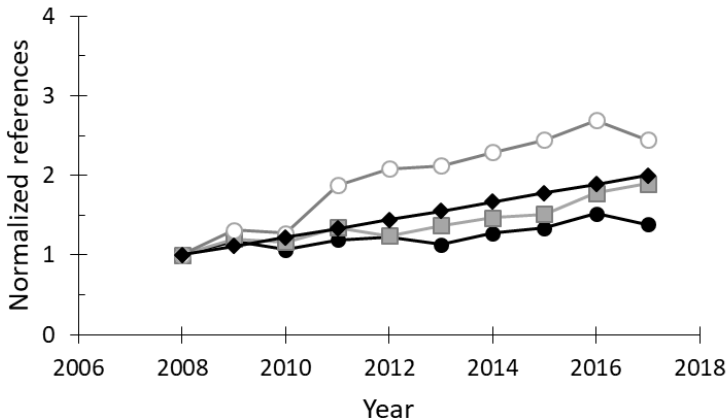
Figure 1. 8. Yearly evolution of the references published in Scopus from 2008 to 2018, normalized to the number of publications in 2008. (♦) Overall scientific articles; (▪) wastewater; (○) wastewater + greenhouse gas; (●) wastewater + membrane bioreactor. Source: Scopus, last access June 2018.

Normalized trends plotted in Figure 1. 8 indicated that the number of both overall scientific articles and articles containing the word "*wastewater*" behaved with a similar trend and growth velocity. Thus, these general topics have been considered as baseline velocities to compare with the other studied items, "*wastewater + greenhouse gas*" and "*wastewater + membrane bioreactor*". When wastewater was combined to membrane bioreactor, it was detected a slight decrease in the number of publications, compared to the reference topics. With regard to MBRs, the great scientific knowledge gained in the last years was enough to currently consider MBRs-based systems as a mature technology. In contrast, a remarkable increase has been noticed when the baseline and "*wastewater + greenhouse gas*" were compared. This result has been according to the current concern of studying new technologies from a holistic point of view. Thus, not only discharge limits were targeted, also carbon footprint and economic studies were needed to make the new system sustainable as a whole.

The number of scientific publications published per year could be represented by an exponential growth function. Worldwide scientific databases have been yearly gaining 8-9% of publications (blogs.nature.com). Thus, considering the available number of papers in 2008, starting point of the current studio, Equation 4 can be used for estimating the annual growth rate of publications in each item of those analyzed when data collected from Scopus is inserted.

$$N = N_0 \cdot (1 + i)^{\Delta n}$$
Equation 4

where:

N: relative number of publications, N_0: number of publications in the first year (2008), Δn: elapsed time in years, i: growth rate.

Iterative estimations were proceeded to obtain the annual growth rates. Results by item were classified in Table 1. 3:

Table 1. 3. Yearly growth rate of publications per item.

Keywords	Growth rate (%)	Source
Worldwide scientific publications	8-9	blogs.nature.com
Wastewater	7.1	Scopus. Calculated.
Wastewater, membrane bioreactor	4.3	Scopus. Calculated.
Wastewater, greenhouse gas	12.8	Scopus. Calculated.

Once the real data were processed and adjusted, the above-predicted behaviors by a qualitative observation were confirmed with a quantitative criterion. Both, overall scientific outputs and wastewater related publications were growing at a similar speed; a yearly 8-9 and 7%; respectively. Researches related to MBR increase in a rate as low as 4.3%. This growth was synergistically pushed up by the increase of the works related to wastewater treatment. As a conclusion, MBR-related velocity decelerated in the last years. On the other hand, a tremendous increase was noted when combining both greenhouse gas and wastewater as fields of interest, confirming the scientific interest of providing a holistic point of view when launching a new technology.

In the period encompassed by this study (2008-2018), as absolute data, a number of 4809 publications were accounted for related to "wastewater, membrane bioreactor". In this term, the number of documents yearly ranged between 276 (in 2008) and 420 (in 2016). Concerning the subject areas in which the publications were included, most of them have been counted in Environmental Science (72%), Chemical Engineering (36%) and Engineering (25%). Journals which collected the highest number of papers were (total number of papers from 2008; CiteScore in 2017): Water Science and Technology (456; 1.34), Bioresource Technology (364; 6.28) and Water Research (338; 7.55).

Geographically, a collection of the publications from 2008 up to nowadays has been summarized in Figure 1. 9.
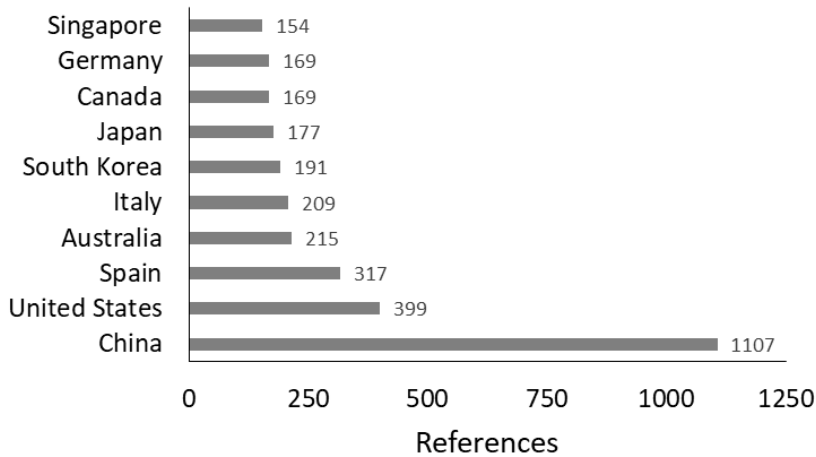
Figure 1. 9. Geographical shares of references with regard to wastewater treatment with membranes in the period 2008-2018 appeared in Scopus (last access June 2018).

The country which accounted for the largest number of publications worldwide has been China (1107), followed by the United States of America (399). The third position hosted Spain (317), being the most important European country in research of wastewater treatment with membranes. Thus, Spain has been one of the reference countries worldwide in gaining knowledge on this topic in the last decade. In the Spanish case, one of the main drivers which concern the scientific community of these countries and encourages to keep investigating in this issue was the growing hydric stress, especially in the Mediterranean/Southern areas and Canary Islands. The lack of water increased policymakers' awareness to invest in wastewater treatments which promote the production of a high quality treated water, enabling a water reuse for many purposes. Within the European framework, Spain was followed by Italy, also with hydric stress issues especially in the Southern area, and Germany. The University of Santiago de Compostela participated in 39 of the overall 317 publications accounted in the period of study, indicating the important relative weight of this institution in the Spanish context.

A comparative study in two different terms, 2008 to 2018 and 2016 to 2018, was also conducted to investigate the evolution of the current topics in the last years and their

possible changes in comparison with the last decade. Those were the keywords inserted in Scopus: fouling, market, reuse, hybrid, aerobic and anaerobic/methane. Figure 1. 10 includes a comparative graph chart between both periods.
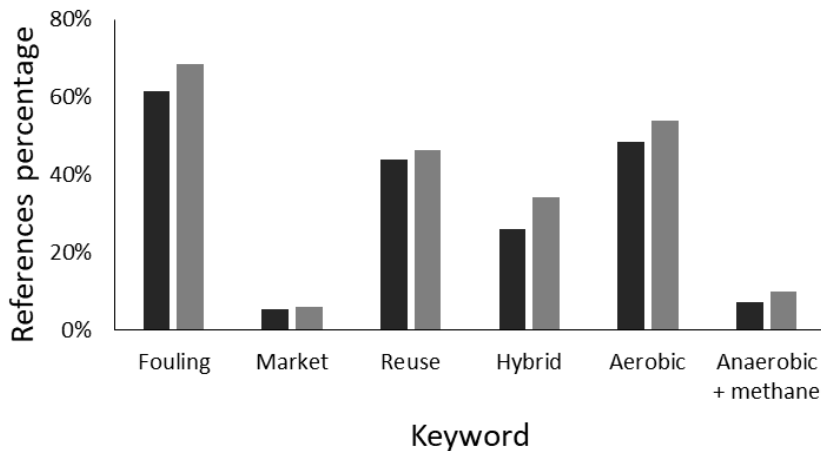

Figure 1. 10. Relative presence of the keywords in the X-axis in the references included in Scopus. Periods: 2016-2018 (black) and 2008-2018 (grey).

In general, only slight differences have been detected when the two studied terms were compared. The keyword "fouling" was a common topic in 2 out of 3 publications with a slim decrease in the last two years. In contrast, "market" was only an important concern in 5-6% of the found references. It indicates that the market is still a topic with a remarkable lack of knowledge and a good field to improve the scientific efforts. On the other hand, in the last years, aerobic and anaerobic systems tended to balance their relative importance. The application of new hybrid flocculent/biofilm biomasses lowered their relative weight in the research level, probably meaning that higher-scale prototypes are being proven.

A complementary study regarding exclusively patents was also conducted. In the reference database "Espacenet" (https://worldwide.espacenet.com; European Patent Office), the words "*membrane bioreactor; wastewater*" have been inserted in the field "*Title or abstract*". This study encompassed the same term than the previous one in Scopus, from 2008 to 2018.

A number of 869 applications and granted patents were published. Figure 1. 11 collects the yearly distribution of these patents over the world.
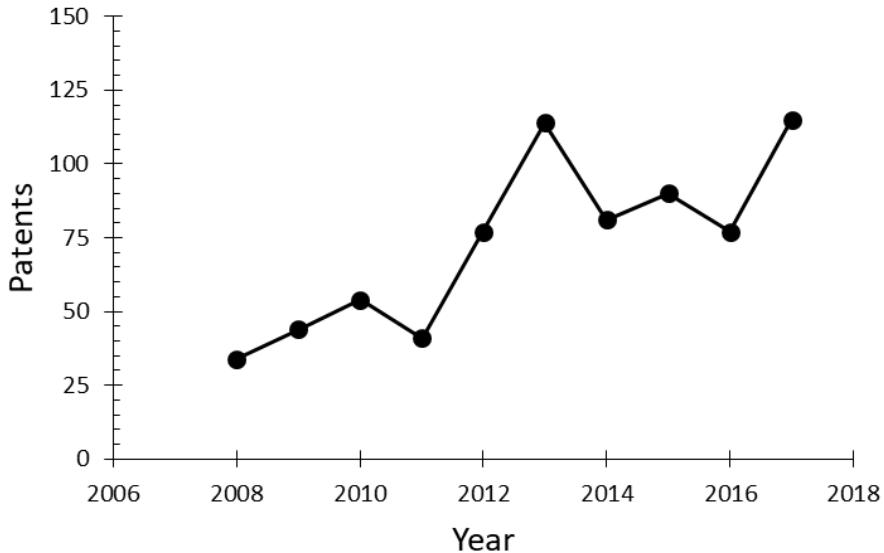

Figure 1. 11. Yearly trends of patents included in the database Espacenet. The inserted keywords were "membrane bioreactor, wastewater".

From 2011 onwards, it was detected a clear interest in developing wastewater treatment technologies with membranes at large scale, excluding 2018 which is not already finished.

Regarding the studied topics, the University of Santiago de Compostela published three patents in the above indicated period. "Anaerobic methanogenic and membrane bioreactor integrated system for the organic matter and nitrogen removal in wastewater, ES 2 401 445 B1 (2014)", "*Three stages membrane biological reactors, methanogenic, aerobic and filtration, for wastewater treatment*, ES 2 385 002 B2 (2013)" and "*Process for the removal of pharmaceutical products present in wastewater*, ES 2 362 298 B2 (2012)".

In conclusion, this bibliometric study related to the scientific references indicated that the interest in the research in wastewater treatment with membranes at scientific scale has been decreasing in the last years. In contrast, other more generalist topics such as wastewater or even science, in general, have been growing at a higher velocity. When joining wastewater with other topics such as the carbon footprint enormous increases in the publishing speed were detected. Thus, combining wastewater treatment with environmental tools can be considered a research hotspot in the last years and forecasts indicated that this behavior will be maintained in the upcoming years.

On the other hand, the above-explained study of patents indicated that the interest in systems which embedded a membrane in the flowchart at large-scale has been gaining attention in the last years. It confirmed that MBRs could be considered as a mature technology and the current interest has been to develop full-scale references instead of researching at lab-scale.

Chapter 2

Materials and methods

Summary

In this chapter, the general analytical methods used in this thesis were described. The most characteristic ones of each part of the research have been also included in the corresponding chapter. Those scientific methods used only during a fraction of this thesis were described in each specific chapter.

Thus, in this Chapter are included: 1) Conventional parameters used for the wastewater in liquid phase: organic matter, nitrogen and phosphorus compounds, pH, dissolved oxygen, solids and carbon compounds concentrations as well as 2) biomass characterization either as suspended solids or as attached biomass and 3) analytical techniques for measuring the composition and the flow of the biogas.

Most of the conventional parameters measured in both the liquid and the solid phase such as chemical oxygen demand (COD), ammonium, nitrite, nitrate, phosphate and mixed liquor (total and volatile) suspended solid (MLTSS and MLVSS) concentrations were determined following Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012). All procedures have been described in detail throughout this chapter. In gas phase it was included the method to quantify biogas composition compounds concentration as long as the generated volume.

As main thread of this PhD thesis, special attention has been given to the membrane's performance describing parameters. Not only conventional ones such as transmembrane pressure, flux or permeability were detailed, also a theoretical explanation of the resistance to filtration determination has been included. Moreover, membrane fouling potential was followed-up by measuring the colloidal biopolymer clusters (cBPC) as a common parameter.

2.1. Liquid phase

In this section, the methods employed for the determination of the conventional parameters of wastewater and sludge have been detailed. For soluble fraction analyses, the samples were previously filtered by using nitrocellulose membrane filters (HA, Millipore) with a pore size of 0.45 μm in order to remove particulate compounds.

2.1.1. Carbon compounds

Chemical oxygen demand

The chemical oxygen demand (COD) is defined as the concentration of a specified oxidant that reacts with the sample under controlled conditions. The quantity oxidant consumed is expressed in terms of its oxygen equivalence. Because of its unique chemical properties, dichromate ion is the specified oxidant. A catalyst (silver sulphate) in acid medium 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 (FAS) to determine the amount of $K_2Cr_2O_7$ consumed, being the amount of oxidizable matter calculated in terms of oxygen equivalent. Both organic and inorganic components of a sample are subject to oxidation, but in most cases the organic content predominates and is of the greater interest (Rice et al., 2012). The total and soluble chemical oxygen demand (COD_t and COD_s) were determined following the method described by Soto et al., (1993), which is a modification from the method 5220C of the Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF in the edition of 1985. The total COD is determined by using the raw sample, while for COD_s determination, the sample was previously filtered through nitrocellulose membrane filters (HA, Millipore) with a pore size of 0.45 μm.

Chemicals used

a) Standard potassium dichromate digestion solution: 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ were dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 were added. The solution was cooled to room temperature and, finally, diluted to 1000 mL. A dilution 1:2 of this solution was used for COD concentration determination below 100 mg·L^{-1}.

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

c) Ferroin indicator solution: 1.485 g of $C_{18}H_8N_2·H_2O$ (phenanthroline monohydrate) and 0.695 g of $SO_4Fe·7H_2O$ were 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 105ºC for 2 hours, were dissolved in 500 mL of distilled water.

e) Standard ferrous ammonium sulphate titrant (FAS) 0.035 N: 13.72 g of $Fe(NH)_4(SO)_2·6H_2O$ were dissolved in distilled water. Then, 20 mL of concentrated H_2SO_4 were added and, finally, the solution was cooled and diluted to 1000 mL. A FAS solution concentration of 0.016 N was used for COD concentration determination below 100 mg·L^{-1}.

Determination procedure

This procedure is applicable to samples with COD concentrations between 90-900 mg·L^{-1}. COD values of 100 mg·L^{-1} or less can be determined by using a more dilute dichromate digestion solution and a more dilute FAS titrant. 2.5 mL of sample were pipetted in 10-mL Pyrex tubes. 1.5 mL of digestion solution and 3.5 mL of sulphuric acid reagent slowly on the wall of the tube slightly inclined (to avoid mixing) were added. A blank sample using distilled water was prepared in the same way. This blank acted as "reference", representing the COD of the distilled water. After being sealed with Teflon and tightly capped, the tubes are finally mixed and placed in the block digester (HACH 16500-100) preheated to 150ºC. The duration of the digestion period was of 2 h.

After digestion, the tubes were cooled down to room temperature. Then, the content of the tubes was transferred to a beaker and, once added 1-2 drops of ferroin indicator, the solution was titrated under rapid stirring with standard FAS. The FAS solution was standardised daily as follows: 5 mL of distilled water into a small beaker were pipetted. 3.5 mL of sulphuric acid reagent added. The mixture was cooled down to room temperature and 5 mL of standard potassium dichromate solution (0.05 N) were

added. 1-2 drops of ferroin indicator and titrate with FAS titrant were added. The end-point is a sharp colour change from blue-green to reddish brown. Molarity of FAS solution is calculated with the following Equation 5:

$$M_{fas} = \frac{5 \cdot 0.05}{V_{fas}}$$ **Equation 5**

where:

M_{fas}: molarity of FAS (mol/L), and

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

The COD is calculated with the following Equation 6:

$$COD = \frac{(A - B) \cdot 8000 \cdot M_{fas}}{V}$$ **Equation 6**

where:

COD: chemical oxygen demand (mg O_2/L),

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.

V: mL of sample

Interferences

The most common interference is the chloride ion. Chloride reacted with silver ion to precipitate silver chloride. This fact resulted in inhibition of the catalytic activity of silver. Bromide and Iodide can interfere similarly.

Total dissolved carbon (TDC), dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC)

The organic carbon in water and wastewater is composed of a variety of organic compounds in different oxidation states. Some of this carbon compounds can be further oxidised by biological or chemical processes and the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) may be used to characterise these fractions. 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 (Rice et al., 2012). 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. In this case, the DOC concentration was measured since the equipment employed only could analyse filtered samples. DOC concentration was determined by a Shimadzu analyser (TOC-5000) as the difference between TDC and DIC concentrations. The instrument was connected to an automated sampler (Shimadzu, ASI-5000-S). The TDC concentrations are determined from the amount of CO_2 produced during the combustion of the sample at 680 °C by using platinum immobilised over alumina spheres as catalyst. The DIC 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 was used as carrier gas with a flow of 150 mL/min. A curve comprising 4 calibration points in the range of 0 to 1 gC/L, using potassium phthalate as standard for TDC and a mixture of sodium carbonate and bicarbonate ($Na_2CO_3/NaHCO_3$, 3:4 w/w) for DIC, was used for the quantification (Figure 2. 1). The detection limit of the equipment is 2 mg/L.
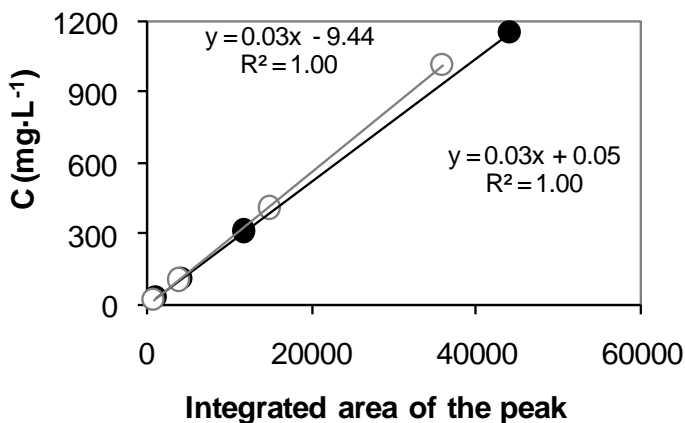
Figure 2. 1. Example of a calibration curve to determine TDC (●) and DIC (○) concentrations.

Volatile fatty acids (VFA)

Volatile fatty acids (VFA) contains a chain of six carbons or fewer, such as acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric, which are intermediate products of the anaerobic digestion. The measurement of VFA concentration has been commonly used as a control test for anaerobic digestion since a VFA accumulation reflects a kinetic disequilibrium between the acids producers and the acids consumers (Switzembaum et al., 1990) and it has been considered a key indicator of process destabilization.

VFA were determined by gas chromatography (HP, 5890A) equipped with a flame ionization detector (FID) and an automatic injector (HP, 7673A). The determination was 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 were 105, 260 and 280°C, respectively. Gas N_2, previously saturated with formic acid before entering into the injector, has been used as carrier gas with a flow of 24 mL/min. Air and H_2 were 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 was made by means of 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. 2). The detection limit of the equipment is 20 mg/L.
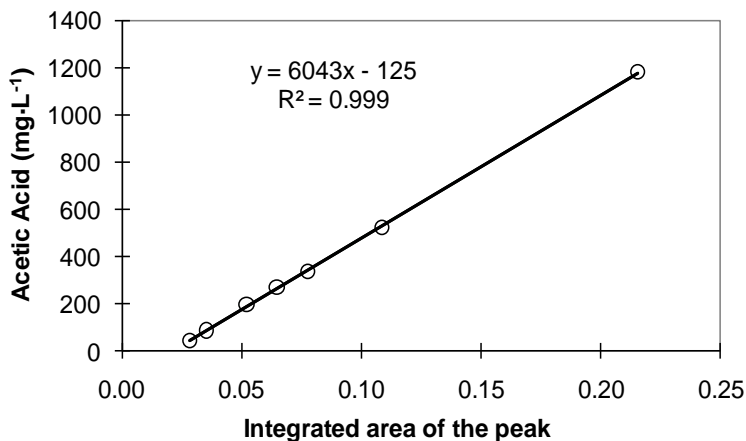

Figure 2. 2. Example of a calibration curve for acetic acid.

2.1.2. Nitrogen compounds

Ammonium by the method of Bower, Holm-Hansen; NH_4^+

Total ammonia-nitrogen ($N-NH_4^+$) was determined spectrophotometrically by a method in which indophenol blue was produced by means of the reaction of ammonia with salicylate and hypochlorite, in the presence of sodium nitroprusside (Bower and Holm-Hansen, 1980). This method substituted phenol–hypochlorite method since phenol was not employed and its application has been considered safer for the executing personnel. The darkness of the appeared blue increased as a function of ammonium concentration.

Reagents:

- Reagent A: Solution of 0.28 g/L of sodium nitroprusside and 440 g/L of sodium salicylate.

- Reagent B: Solution of 18.5 g L^{-1} of NaOH and 120 g/L of sodium citrate.

- Reagent C: Standard commercial solution of sodium hypochlorite.

- Reagent D: Solution prepared mixing 7 parts of reagent B and 1 part of reagent C. Reagent D was stable for 1 hour after preparation.

Determination Procedure:

- Add 120 µL of reagent A and 200 µL of reagent D to 1 mL of sample (diluted if necessary).

- Store, protected from light, between 2 and 3 hours.

- Measure the absorbance at 640 nm and compare with the calibration curve (Figure 2. 3) which represents ammonia concentration as a function of the absorbance at 640 nm.
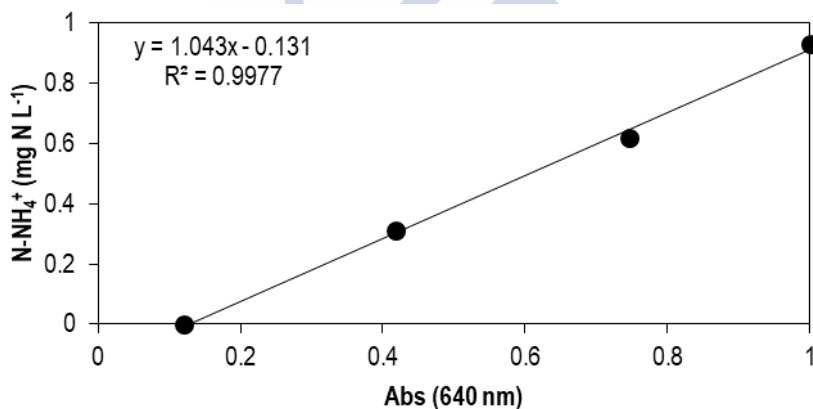

$y = 1.043x - 0.131$
$R^2 = 0.9977$

Figure 2. 3. Calibration curve for ammonia determination by the Bower method.

Nitrite, NO_2^-

Nitrite concentration in wastewater was determined following the method included in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012).

Nitrite is determined through the formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized sulphanilamide with N-(1-napththyl)-ethylenediamine dihydrochloride (NED dihydrochloride). The applicable range of the method for spectrophotometric measurements is 0 to 0.3 $mgN\text{-}NO_2^-/L$.

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 is 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), it should be added 0.1 mL of each solution (sulphanilamide and NED). After waiting for 20 min for colour stabilisation, the sample has been measured in a spectrophotometer (Cecil CE 7200) at a wavelength of 543 nm. The quantification has been carried out by means of a 8-10 points calibration curve in the range of 0-0.25 mg $N-NO_2^-/L$, using $NaNO_2$ as standard (Figure 2. 4).

The graph shows N-NO$_2^-$ (mg N L^{-1}) versus Abs. (543 nm), with fit $y = 0.3021x - 0.0022$ and $R^2 = 0.9995$.
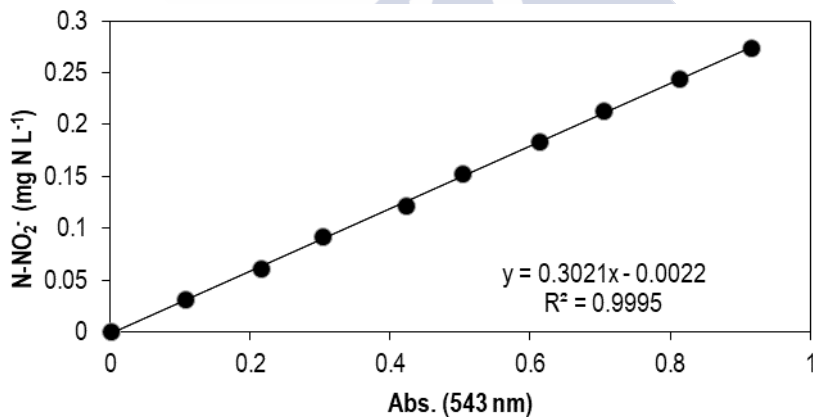
Figure 2. 4. Example of a calibration curve for nitrite concentration determination.

Interferences

Chemical incompatibility makes it unlikely that NO_2^-, free chlorine and nitrogen trichloride (NCl_3) will coexist. NCl_3 imparts a false red colour when colour reagent is added. The following ions interfere because of precipitation under test conditions and

should be absent: Sb^{3+}, Au^{3+}, Bi^{3+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^+, chloroplatine and metavanadate. Moreover, cupric ion may cause low results by catalysing decomposition of the diazonium salt.

The determination should be promptly made on fresh samples in order to avoid bacterial conversions of NO_2^-. At least filtration of the samples should be conducted immediately after collection.

Nitrate, NO_3^-

Nitrate concentration in wastewater was determined following the method 4500-NO_3^--B described in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012).

Measurement of UV absorption at 220 nm enabled rapid determination of NO_3^- ions. Because dissolved organic matter also may absorb at 220 nm and NO_3^- did not absorb at 275 nm, a second measurement at 275 nm was used to correct the NO_3^- value. If correction value is more than 10% of the reading at 220 nm, this method should be substituted.

Determination procedure

Place 5 mL of sample (diluted if necessary to get a maximum concentration of N-NO_3^- of 2.5 mg·L^{-1}) and add 0.1 mL of HCl 1N. Afterwards, the absorbance at 220 and 275 nm was measured in a spectrophotometer (Cecil CE 7200) with quartz or matched silica cells of 1 cm or larger light path. The absorbance related to nitrate was obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm according to Equation 7. The quantification was carried out by a 8-10 points calibration curve in the range of 0-17.5 mg N-NO_3^- ·L^{-1}, using KNO_3 as standard (Figure 2.5).

$$mgN - NO_3^- \cdot L^{-1} = a \cdot (A_{220nm} - 2 \cdot A_{275nm}) + b \quad \text{Equation 7}$$

where A_{220nm} and A_{275nm} are the absorbances at 220 and 275 nm, respectively,

a is the slope of the calibration curve and

b is the intercept.

Interferences

Dissolved organic matter, surfactants, NO_2^- and Cr^{6+} interfere with NO_3^- determination. Moreover, various inorganic ions such as chlorite and chlorate may interfere. The determination should be promptly made on fresh samples in order to avoid bacterial conversions of NO_2^-. At least filtration of the samples should be performed immediately after the samples collection. For longer storage of unchlorinated samples (more than two days), preserve with 2 mL conc. $H_2SO_4 \cdot (98\%)$ and store at 4 ºC (fridge). It should be noticed that when sample is preserved with acid, NO_3^- and NO_2^- cannot be determined as single species.
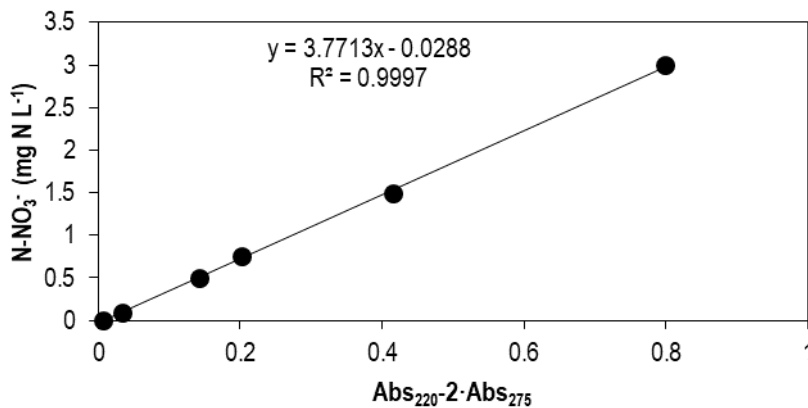

Figure 2. 5. Example of a calibration curve for nitrate concentration determination.

Dissolved total nitrogen (DTN), dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN)

DTN was determined in a total organic nitrogen analyzer (Rosemount-Dohrmann DN-1900) equipped with a quimioluminiscence detector with two channels. One channel determines the DTN, by oxidation at high temperature, and the other determines the DIN, by a chemical reduction. DON is determined as the difference between DTN and DIN.

All the nitrogen present in the water is catalytically oxidised to nitrous oxide (NO). The process for DTN 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 80ºC and catalyzed by $VaCl_3$. For the DIN 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 DTN and DIN is carried out by quimioluminiscence, using a multiplicator tube. The instrument is calibrated with a certified standard solution (KNO_3, 20 mgN/L) using a response factor method.

2.1.3.   Phosphorus compounds

Orthophosphates

Orthophosphate concentration in wastewater was determined following the method 4500-P-E described in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012).

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

Reagents preparation

Reagent A: Sulphuric acid 5N.

Reagent B: Solution of antimony potassium tartrate. 1.3715 g of $K(SbO)C_4H_4O_6 \cdot 0.5H_2O$ were 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 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ were 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. This solution is stable for only one week.

Combined reagent: To prepare 100 mL of the combined reagent, the reagents A to D were mixed according to the following volumes: 50 mL of reagent A, 5 mL of reagent B, 15 mL of reagent C and 30 mL of reagent D. The mixture must be stirred after the

addition of each reagent. It should be noticed that the above mentioned order has to be maintained. This combined reagent is stable for only 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) was added. If red color appears, reagent A (H_2SO_4 5N) was added (drops) until the red color disappears. Then, 0.8 mL of the combined reagent was added and the mixture was stirred with a vortex stirrer. After 10 minutes but before 30 minutes, the absorbance at 880 nm has been measured with a spectrophotometer Cecil CE 7200. The quantification was performed by means of a 6-8 points calibration curve in the range of 0-1 mg $P\text{-}PO_4^{3-}$/L, using KH_2PO_4 as standard (Figure 2. 6).

Interferences

Concentrations of arsenates as low as 0.1 mg/L reacted with the molybdate reagent to produce a blue color similar to that formed with phosphate. Hexavalent chromium and NO_2^- interfere to give results about 3% low at concentrations of 1 mg·L^{-1} and 10 to 15% low at 10 mg/L. Filtration of the samples should be carried out immediately after collection.

Total phosphorus

Because phosphorus may occur in combination with organic matter, 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 was taken and one drop of phenolphthalein indicator solution was added. If red color appears, some drops of reagent A (H_2SO_4 5N) were 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 $(NH_4)_2S_2O_8$ were added. The mixture was gently boiled in an electric 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 was cooled and diluted to 30 mL with distilled water. A drop of phenolphthalein indicator solution was added and the mixture

was neutralized with NaOH 1N till pale pink color was observed. Then the phosphorus concentration was determined with the colorimetric method previously described for orthophosphate.
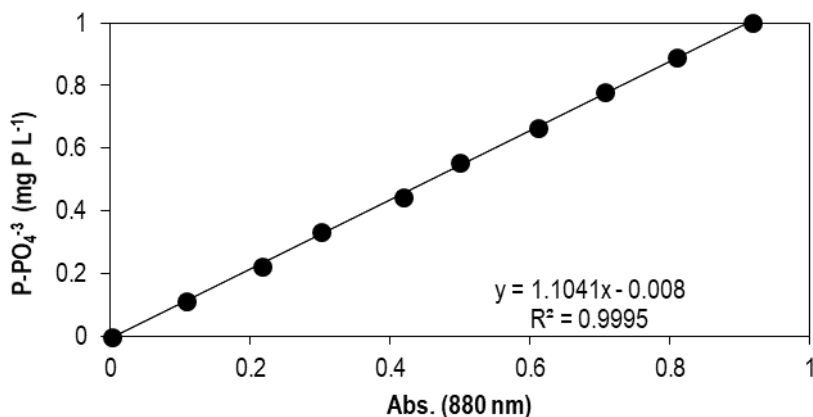

Figure 2. 6. Example of a calibration curve for orthophosphate concentration determination.

2.1.4. Other control parameters

pH

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 (Crison Instruments, S.A., 52-03). The sensibility of the instrument is ± 1 mV, corresponding to 0.01 pH units. The electrodes are calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

Dissolved oxygen and temperature

Temperature and dissolved oxygen (DO) were measured with a multi-parameter meter (Hach HQ40d) with a luminescent optical probe (IntelliCAL LDO101).

Alkalinity and alkalinity ratio

Wastewater alkalinity of water is its neutralization capacity of the contained acids. It is the sum of all the titratable bases and its value vary significantly depending on the end-point pH used. Alkalinity is significant in many uses and treatments of natural waters and wastewaters. Because the alkalinity of many surface waters is a primarily function of carbonate, bicarbonate and hydroxide concentration. The measured values may include contributions from borates, phosphates, silicates, or other bases. Alkalinity measurements have been used in the interpretation and control of water and wastewater treatment process, such as anaerobic digestion. A typical symptom of a non-desirable operation of an anaerobic reactor is the increase of the organic acids concentration, which occurs when their production exceeds their consumption. This latter behavior has been typically followed up by means of the alkalinity measurement.

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

Various authors established that the relation between IA and TA has been an adequate parameter for controlling the stability of anaerobic digestion process. It should not exceed the value of 0.3 (Ripley et al., 1986; Soto et al., 1993; Switzembaum et al., 1990; Wentzel et al., 1994) to avoid the accumulation of the VFA in the system.

Determination of the alkalinity was performed according to the method 2320 of Rice et al. (2012) 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 $mgCaCO_3/L$ and are calculated as follows (Rice et al., 2012):

$$PA = A \cdot N \cdot 50000/V \qquad \text{Equation 8}$$

$$TA = B \cdot N \cdot 50000/V$$

Equation 9

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.

2.2. Quantification of solids and sludge settleability

2.2.1. Mixed liquor total suspended solids (MLTSS) and mixed liquor volatile suspended solids (MLVSS) concentration

Suspended solids content in water can be organic or inorganic. Mixed liquor total solids (MLTS) is the term applied to the material residue left in the vessel after evaporation of a sample and its subsequent drying in an oven at a defined temperature. MLTSS includes mixed liquor total suspended solids (MLTSS), the portion of MLTS retained by a filter, and dissolved solids, the portion that passes thorough the filter. Mixed liquor volatile solids (MLVS) and mixed liquor volatile suspended solids (MLVSS) are the fraction of MLTS and MLTSS, respectively, which are loss on ignition at a specified temperature. The determination of MLVSS concentration is especially useful in the control of wastewater treatment plant operation because it offers a rough approximation of the amount of organic matter present in the solid fraction of wastewater, activated sludge or industrial wastes. MLTS and MLTSS are determined following the methods described in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012).

Determination procedure

MLTS are determined weighing a selected (in order to yield a residue between 2.5 and 200 mg) well-mixed sample volume in a previously clean (heated to 103-105ºC for 2 h) porcelain cup after being evaporated at 103-105ºC until constant weight. The

increase in weight over that of the empty dish represents the total solids in the initial volume of sample.

For the determination of MLTSS, a selected (in order to yield a residue between 2.5 and 200 mg) well-mixed sample volume is filtered through a weighed glassfiber 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 103-105ºC. The increase in weight of the filter represents the total suspended solids.

To determine the volatile solids (MLVS or MLVSS), the residue is burnt to constant weight at 550ºC during half an hour. The weight lost during ignition corresponds to the volatile solids.

Interferences

Highly mineralized water with a significant concentration of calcium, magnesium, chloride and/or sulphate may be hygroscopic and requires prolonged drying, proper desiccation and rapid weighing. Some inorganic salts such as hydroxides, carbonates or salts of ammonia were decomposed and volatilised at 550 ºC and therefore can give a higher value for the volatile content in the sample.

2.2.2. Biofilm concentration

The type of biofilm carrier was a foamy support characterized by its remarkable porosity and its soft touch. Nevertheless, these main characteristics made this method unsuitable for applying the traditional method by means of sonication. A gravimetry method to estimate the concentration of solids attached onto the walls was developed. It consisted of drying 25 pieces of carrier in the oven at 105ºC for at least 12 h. After dried, the overall mass was measured by weighting the carriers.

In order to estimate the average solids concentration, the above calculated mass with attached biomass was compared with the mass of the same number of new carriers. Then the following equation was employed to estimate the mass for each piece of carrier.

$$TA = B \cdot N \cdot 50000/V$$

Equation 10

where:

$m_{carrier} \equiv$ mass of the set of 25 Levapor carriers,

$m_{105ºC} \equiv$ mass of the set of 25 Levapor carriers after being dried at 105ºC for at least 12 h,

$m_{new} \equiv$ mass of a set of 25 new Levapor carriers,

$N \equiv$ number of compared carriers, normally 25.

2.2.3. Sludge volumetric index

The sludge volumetric index (SVI) determination is defined in the Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012) as the volume in mL, occupied by 1 g of MLTSS after 30 min settling.

2.2.4. Sludge filterability

This method has been explained in detail on Thiemig, (2012). The aim was to measure the time to filter the sludge by separating by gravity solid and liquid phases through a commercial filter. Moreover, the last calculation consisted of referring the time to the total solids concentration (MLTSS). A schematic of the assembly was depicted in Figure 2. 7.
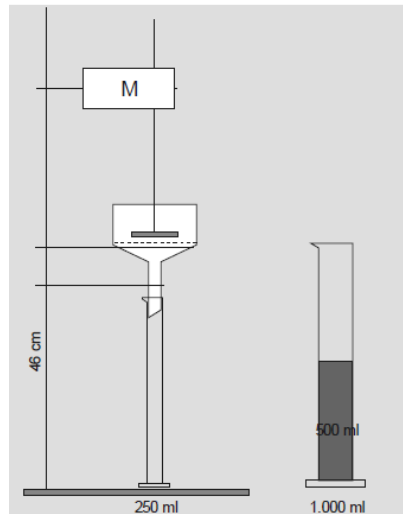
Figure 2. 7. Schematic of the assembly needed for conducting the sludge filterability index (SFI) method.

Once the value was obtained it can be compared with other previous obtained or reported values in order to determine the actual state of the sludge.

2.3. Gas phase

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

Biogas production was measured using a Milli GasCounter MGC-10 (Ritter, Germany), which basically consists in a tilting body inside a container with a special packing liquid. The entrance of gas bubbles led the tilting body to change its position. Each change is counted with a magnet and a counter and the internal calibration give the gas flow in the display.

Other methods as the employed to determine dissolved methane in liquid phase were included in the specific Chapter 5.

2.4. Membrane performance

2.4.1. Flux and permeability

Membrane flux can be calculated as:

$$J = \frac{Q}{A}$$

Equation 11

where:

J: flux of permeate expressed in [L/(m²·h)],

Q: flow expressed in L/h,

A: membrane area expressed in m².

Therefore, permeability can be calculated as:

$$P = \frac{J}{TMP}$$

Equation 12

where:

P: permeability expressed in [L/(m²·h·bar)],

TMP: transmembrane pressure in [bar].

2.4.2. Critical flux

The critical flux hypothesis is that on start-up there exists a flux below which a decline of flux with time does not occur; above it fouling is observed. This flux is the critical flux and its value depends on the hydrodynamics and probably other variables. The critical flux was determined according to the 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).

2.4.3. Filterability

The specific resistance to filtration of a sludge sample was determined by a dead-end filterability test. The test was conducted at 25ºC in a 180 mL stirred cell (Model 8200, Amicon) using a 0.45 µm flat-sheet PVDF membrane filter of (HVLP 09050, Millipore) in a 200 mL pressurized cylinder (Model Sartorius SM 16249) using a 0.2 µm flat-sheet cellulose acetate membrane filter (12587-47-N Sartorius). The stirred cell and the cylinder were filled with 180 mL of the sample liquor and a constant pressure was applied by pressurized nitrogen. The production of filtrate under pressure was continuously recorded by an electric balance (Sartorius BP 1200) that was connected to a computer.

The resistance-in-series model was applied to evaluate the filtration characteristics.

$$J = \frac{\Delta P}{\eta \cdot R_t} \qquad \qquad \text{Equation 13}$$

$$R_t = R_m + R_c + R_{pb} \qquad \qquad \text{Equation 14}$$

Where J is the permeation flux [m³/(m²·s)], ΔP is the TMP [Pa], η is the dynamic viscosity of the permeate [Pa/s]; R_t is the total resistance [m⁻¹]; R_m is the intrinsic membrane resistance [m⁻¹]; R_c is the cake resistance formed by the cake layer deposited over the membrane surface [m⁻¹]; and the pore blocking resistance, R_{pb}, is the resistance caused by solute adsorption into the membrane pores and walls [m⁻¹]. Each resistance value can be obtained through the Equation 15, Equation 16, Equation 17:

$$R_m = \frac{\Delta P}{\eta \cdot J_m} \qquad \qquad \text{Equation 15}$$

$$R_{pb} = \frac{\Delta P}{\eta \cdot J_{pb}} - R_m \qquad \qquad \text{Equation 16}$$

$$R_c = \frac{\Delta P}{\eta \cdot J} - (R_m + R_{pb})$$

Equation 17

The experimental procedure to determine each resistance value was as follows: (a) R_m was estimated by measuring the permeate flux of tap water; (b) R_t was evaluated by the flux of biomass microfiltration; (c) the membrane surface was then flushed with tap water and cleaned with a sponge to remove the cake layer. After that, the tap water flux was measured again to obtain the resistance of $R_m + R_{pb}$. From steps (a)–(c), R_t, R_m, R_{pb} and R_c could be calculated. The resistance of the colloidal fraction of the cake was also determined using a new filter according to Equation 18:

$$R_{col} = \frac{\Delta P}{\eta \cdot J_{col}} - R_m$$

Equation 18

where J_{col} is the flux of the supernanatant after centrifugation of biomass at 4000 g during 10 min.Using the Carman-Kozeny equation to calculate the pressure drop of a fluid flowing through a packed bed of solids in laminar flow and taking into account that the filtration took place at constant pressure, the specific resistance to filtration (SRF) (α, m/kg) was calculated after linearization according Equation 19:

$$\alpha = \frac{2 \cdot A^2 \cdot P \cdot b}{\eta \cdot w}$$

Equation 19

where P is the applied pressure [Pa], A the filtration area [m²], w the total suspended solids [kg/m³], η is the dynamic viscosity of filtrate [Pa·s] and b is the time-to-filtration ratio [s/m⁶], which is the slope of the curve that is obtained by plotting the time of filtration to the volume of filtrate ratio (t/V) versus the filtrate volume (V). From the conventional constant pressure filtration equation, a plot of t/V vs. V is expected to yield a linear relationship for the entire filtration data. The linearity of t/V vs. V plot is observed only when the value of V (or time) or the cake thickness is sufficiently large.

2.4.4.   Colloidal biopolymer clusters (cBPC)

A pool of biopolymer clusters (BPC) ranging from 2.5 to 60 µm in size was identified in the liquid phase of the MBR sludge and in the cake sludge on the membrane surface. BPC are free and independent organic solutes that are different from other types of fouling substances such as EPS and SMP (Sun et al., 2008). It was previously found

that the colloidal fraction of the BPC (cBPC) was easier measurable and the results were strongly related with the actual BPC (Sánchez et al., 2013). The difference in tDOC concentration between the sludge mixture after filtration through a 0.45 µm nitrocellulose membrane filter (HA, Millipore) and the permeate was assigned to the cBPC in the liquid phase of the sludge mixture suspension. Concentration of total dissolved organic carbon (tDOC) was measured with a Shimadzu analyser (TOC-5000).

2.4.5. Transparent exopolymer particles (TEP)

The method of analysis method used for the determination of the TEP concentrations (De La Torre et al., 2008) has been based on the protocol developed for TEP quantification in sea water (Arruda-Fatibello et al., 2004). The former consists of mixing 5 mL of prefiltered sample with 0.5 mL of 0.055% (m/v) alcian blue solution and 4.5mL of 0.2 mol/L acetate buffer solution (pH 4) in a flask. The flask was then stirred for 1 min and then centrifuged (Centrifuge MR23i Jouan GmbH, Germany) at 15300 rpm for 10 min. TEP react with the alcian blue solution yielding a low solubility dye–TEP complex. The concentration of the alcian blue in excess is determined by reading the absorbance at 602 nm (UV-vis spectrophotometer, Analytic Jena, Germany). The quantification was carried out by means of a 6-8 points calibration curve in the range of 0-250 mg/L, using xanthan gum (XG) (Figure 2. 8). The results expressed in mg/L xanthan gum equivalent.
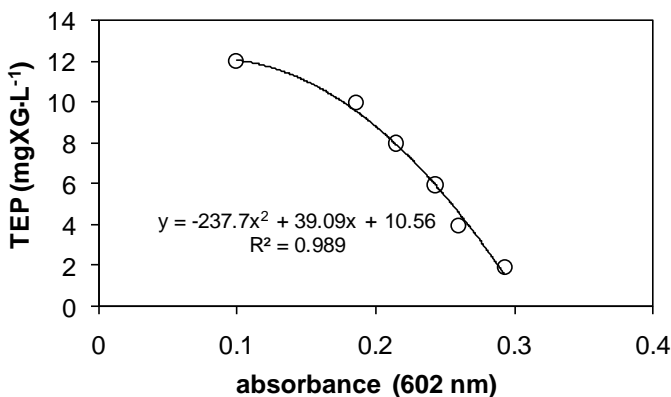

The plot shows TEP (mgXG·L⁻¹) versus absorbance (602 nm) with the fitted equation:

$$y = -237.7x^2 + 39.09x + 10.56$$
$$R^2 = 0.989$$

Figure 2. 8. Calibration curve for TEP concentration determination.

Other membrane fouling parameters or their adaptation to specific situations were included in Chapters 3, 4, and 5.

2.5.   Membrane cleaning procedures

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

2.5.1.   Maintenance cleaning

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

1) physical cleaning by rinsing with tap water, and

2) backwashing with chlorinated water (250-500 ppm Cl_2) for 1 h.

2.5.2.   Intensive Chemical Cleaning

Chemical cleaning was performed outside the membrane chamber only when permeability value was below 50 L/(m^2·h·bar), approximately. The cleaning procedure was:

1) physical cleaning by rinsing with tap water,

2) Submerging the membrane in chlorinated water (500 ppm Cl_2) for 8 h.

Other protocols were described in Chapters 3 and 4 due to the specificities of the research.

2.6.   Microbiological determinations

Molecular techniques based on the rRNA of *Bacteria* and *Prokaryotes* are presented in the next section. It is the most commonly employed quantitative molecular biology technique, although quantification is either complex or tedious and a bit subjective. Even so, it has been considered a very useful tool in the field of Environmental Engineering.

FISH technique targets the rRNA of the microorganisms even until the taxonomical level of individual, depending on the existence of the probe and the required specificity.

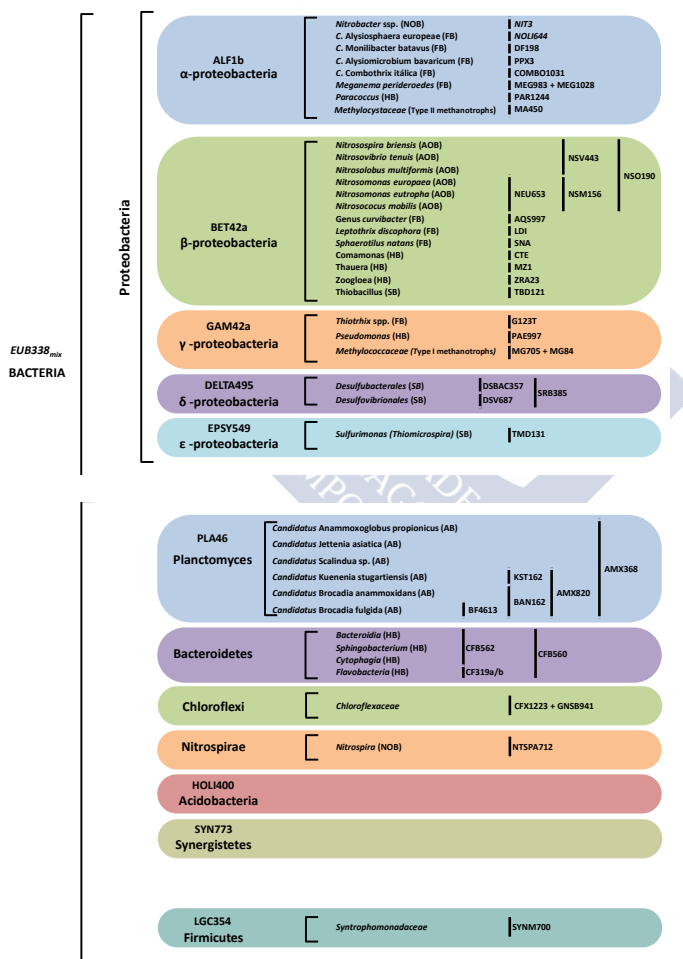### 2.6.1. Identification of bacteria populations by FISH

The abundance of the different populations of microorganisms presented in the sludge samples of the reactors has been investigated by means of FISH. With this technique, specific regions in the 23S or 16S rRNA were targeted with labelled probes which shined in the presence of fluorescent light. If the corresponding domain, phylum, genus or species are present, the probe hybridizes to the targeted sequence and can be detected by means of microscopy. According to Amann et al., (1995) a typical FISH protocol includes four steps: I) fixation and permeabilization of the sample; II) hybridization of the targeted sequence to the probe; III) washing steps to remove the unbound probe; and finally, IV) 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 was sonicated for 1 minute at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher). The time of sonication was selected in order to achieve the breakage of the granules but not of the cells.

During hybridization, the cells were exposed to high temperatures, detergents and osmotic gradients. Thus, fixation of the cells was essential in order to maintain the morphological integrity of the cells. Fixation of cells with glutaraldehide resulted in considerable auto fluorescence of the specimen. Auto fluorescence was minimized by fixation in freshly prepared (not older than 24 h) 4% paraformaldehyde solution in phosphate buffer solution (PBS).

After fixation, the cells were 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 was done at stringent conditions (46 ºC, 0-65% formamide) and specimens were washed with wash buffer (48 ºC). The targeted organisms can be detected by the characteristic fluorescence of the dye contained in the probe.

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 phylogenetic tree reflecting different probes study indicating the bacteria detected by each probe are shown in Figure 2.1. Those probes applied in this study are listed and detailed in Table 2. 1.
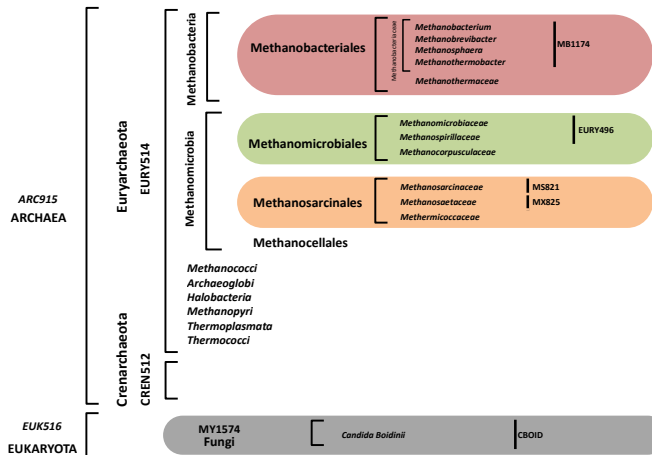
EUB338$_{mix}$ BACTERIA

Proteobacteria

ALF1b α-proteobacteria
- *Nitrobacter* ssp. (NOB) — NIT3
- *C. Alysiosphaera europeae* (FB) — NOLI644
- *C. Monilibacter batavus* (FB) — DF198
- *C. Alysiomicrobium bavaricum* (FB) — PPX3
- *C. Combothrix itálica* (FB) — COMBO1031
- *Meganema perideroedes* (FB) — MEG983 + MEG1028
- *Paracoccus* (HB) — PAR1244
- *Methylocystaceae* (Type II methanotrophs) — MA450

BET42a β-proteobacteria
- *Nitrosospira briensis* (AOB)
- *Nitrosovibrio tenuis* (AOB) — NSV443
- *Nitrosolobus multiformis* (AOB)
- *Nitrosomonas europaea* (AOB)
- *Nitrosomonas eutropha* (AOB) — NEU653 — NSM156 — NSO190
- *Nitrosococcus mobilis* (AOB)
- Genus *curvibacter* (FB) — AQS997
- *Leptothrix discophora* (FB) — LDI
- *Sphaerotilus natans* (FB) — SNA
- *Comamonas* (HB) — CTE
- *Thauera* (HB) — MZ1
- *Zoogloea* (HB) — ZRA23
- *Thiobacillus* (SB) — TBD121

GAM42a γ-proteobacteria
- *Thiothrix* spp. (FB) — G123T
- *Pseudomonas* (HB) — PAE997
- *Methylococcaceae* (Type I methanotrophs) — MG705 + MG84

DELTA495 δ-proteobacteria
- *Desulfubacterales* (SB) — DSBAC357 — SRB385
- *Desulfovibrionales* (SB) — DSV687

EPSY549 ε-proteobacteria
- *Sulfurimonas (Thiomicrospira)* (SB) — TMD131

PLA46 Planctomyces
- *Candidatus* Anammoxoglobus propionicus (AB)
- *Candidatus* Jettenia asiatica (AB)
- *Candidatus* Scalindua sp. (AB)
- *Candidatus* Kuenenia stugartiensis (AB) — KST162 — AMX368
- *Candidatus* Brocadia anammoxidans (AB) — BAN162 — AMX820
- *Candidatus* Brocadia fulgida (AB) — BF4613

Bacteroidetes
- *Bacteroidia* (HB)
- *Sphingobacterium* (HB) — CFB562 — CFB560
- *Cytophagia* (HB)
- *Flavobacteria* (HB) — CF319a/b

Chloroflexi
- *Chloroflexaceae* — CFX1223 + GNSB941

Nitrospirae
- *Nitrospira* (NOB) — NTSPA712

HOLI400 Acidobacteria

SYN773 Synergistetes

LGC354 Firmicutes
- *Syntrophomonadaceae* — SYNM700

Figure 2.1. FISH probes and the main bacteria detected by each probe.

The three probes for the domain of eubacteria (EUB338, EUB338 II and EUB338 III) were applied together in all samples to get an impression of the relative abundance of the microorganisms detected by more specific probes. In comparison with DAPI, they provided evidence of non-eubacteria present in the sample. 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 as well. Still this error it was tried to be kept small.

Table 2. 1. Probes used for fluorescent in situ hybridization and the formamide (FA) concentration used during hybridization.

Probe	Probe sequence (5'→3')	% FA	Target organisms	Ref.
EUB 338	GCTGCCTCCCG TAGGAGT	0-50	Bacteria domain	[1]
EUB 338 II	GCAGCCACCCG TAGGTGT	0-50	*Planctomycetales*	[2]
EUB 338 III	GCTGCCACCCG TAGGTGT	0-50	*Verrucomicrobiales*	[2]
NSO1225	CGCCATTGTATT ACGTGTGA	35	*Ammonio-oxidizing-β-Proteobacteria*	[4]
NTSPA0662	GGAATTCCGCG CTCCTCT	35	*Nitrospira*	[5]
NTSPA712 Competitor	CGCCTTCGCCA CCGGCCTTCC	35	Most members of phylum Nitrospira	[5]
AMX 368	CCTTTCGGGCA TTGCGAA	15	All anammox bacteria	[6]
AMX 820	AAAACCCCTCTA CTTAGTGCCC	30-40	Anammox	[16]
MG 84	CCACTCGTCAG CGCCCGA	20	Methanotrophs type I	[17]
MG 705	CTGGTGTTCCTT CAGATC	20	Methanotrophs type I	[17]
MA 450	ATCCAGGTACC GTCCATTATC	20	Methanotrophs type II	[17]
DBACT 193	CGCTCGCCCCC TTTGGT	45	DAMO bacteria	[18]
DBACT 1027	TCTCCACGCTC CCTTGCG	40	DAMO bacteria	[18]
DARCH872	GGCTCCACCCG TTGTAGT	40	DAMO archaea	[15]

[a] **References: [1] Amann et al., (1990); [2] Daims et al., (1999); [3] Manz et al., (1992); [4] Mobarry et al., (1996); [5] Daims et al., (2001); [6] Schmid et al., (2003); [7] O`Sullivan et al., (2001); [8] Lajoie et al., (2000); [9] Schleifer et al., (1992); [10] Rosselló-Mora et al., (1995); [11] Crocetti et al., (2000); [12] Gich et al., (2001); [13] Björnsson et al., (2002); [14] Raskin et al., (1994); [15] Stahl and Amann (1991);[16] Schmid et al.,[17] Eller et al. (2001); [18] Raghoebarsing et al. (2006)**

2.6.2. Quantification

Quantification of the bacterial population was based on the use of daime (digital image analysis in microbial ecology) software by measuring the relative abundances (fractions of the total biovolume) of probe labelled populations in digital images (Daims et al., 2006). The quantification was performed by comparison of the positive area obtained with a specific probe with the area corresponding to the control: DAPI or EUBmix (a mixture of EUB338, EUB338 II and EUB338 III). Digital images from 20 to 30 different fields of view were obtained at randomly chosen positions.

This program does not quantify absolute cell numbers, but determines the biovolume fraction of the specifically labelled target population relative to the biovolume of the total biomass. Although one of the recommendations of daime software is its use with images acquired by using a confocal microscope, in this thesis, daime software was used with images acquired by using an epifluorescence microscope, with the objective of having an approximate idea of the percentages of certain populations.

2.6.3. Reagents preparation

- PBS (3x): An amount of 0.49 g KH_2PO_4 was dissolved in 80 mL of milliQ water, then 2.3 g of NaCl were added and the pH value was adjusted to 7.2. Finally, the volume was adjusted to 100 mL.
- PBS (1x) was prepared by a 1:3 dilution of PBS (3x) in milliQ water.
- Fixative solution: First, 6.5 mL milliQ were heated to 60 °C and 0.4 g of paraformaldehyde were added to them. One drop of 1 M NaOH was added and the solution was shaken vigorously until it had nearly clarified (1-2 min). Then, 3.3 mL of PBS (3x) were added and the pH was adjusted to 7.2 with HCl (one drop 1 M HCl). Finally, the solution was filtered through 0.2 μm membrane filter.
- Hybridization buffer: The buffer was prepared into a 2 mL eppendorf by mixing: 360 μL of NaCl 5 M and 40 μL of Tris/HCl (1 M) (pH 8.0). The percentage of formamide of the hybridization buffer was selected according to the used probe (% FA in Table 2. 2). Finally, 4 μL of sodiumdodecylsulfate 10% (w/v) were added to the mixture

Table 2. 2. Formamide and water added to the hybridization buffer.

% Formamide (v/v)	Formamide (µL)	MilliQ (µL)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400

Other protocols regarding microbiological aspects have been included in the Chapter 6 carried out in the Radboud Universiteit Nijmegen (The Netherlands).

Chapter 3

Performance of a hybrid membrane bioreactor treating a low strength and alkalinity wastewater[1]

Summary

A pilot-scale Hybrid Membrane Bioreactor (HMBR) containing both suspended biomass and biofilm was tested for the treatment of a low strength municipal wastewater. The wastewater fed was characterized by a high variability throughout the day, low BOD_5/TN ratio and low alkalinity (302 ± 52 $mgCaCO_3/L$). For limiting membrane fouling, an innovative abrasive granular material has been proven in the Microdyn-Nadir membrane. Permeability ranged from 126 to 291 $L/(h\ m^2\ bar)$ during the operational period, achieving a maximum flux of 24 $L/(m^2\ h)$. A low BOD_5/TN ratio of the raw wastewater, led to insufficient denitrification, with an average nitrogen removal of 49%. This fact, in turn, caused a decrease in the pH due to the lack of alkalinity. This study underlined that wastewaters characterized by high variability throughout the day, low BOD_5/TN ratio and/or low alkalinity content require carefully design of the MBR systems. It was shown that a low pH in the HMBR led to a strong membrane fouling increasing cake resistances.

3.1. Introduction

Over the last century, conventional activated sludge systems (CAS) have been spread all over the world in municipal wastewater treatment plants (WWTP) because of its reliability and the long experience accumulated. In comparison with other newly developed biological technologies, such as membranes, aerobic granular sludge, or biofilm reactors (based on attached biomass), CAS facilities presented lower removal organic loading rates (OLR) (Metcalf&Eddy et al., 2014).

Membrane technologies have become a well-acknowledged treatment process implemented worldwide for wastewater treatment (Siembida et al., 2010). A membrane bioreactor (MBR) can be defined as a modified conventional activated sludge (CAS) reactor with a membrane filtration process instead of the secondary settler (Iglesias-Obelleiro et al., 2012). MBR-related technologies offer many advantages over the CAS processes. The quality of the effluent is better, especially in terms of suspended solids and microbial indicators (Judd, 2008) in comparison with the CAS outputs. Moreover, the footprint requirements of MBR and the improved capacity of controlling the applied solids retention time (SRT) have made this technology very attractive. For instance, MBR systems are recommended when land scarcity is an issue or for treating sewage in areas with high environmental sensitivity.

The energy demand of MBR processes is still higher than that of CAS but similar to those facilities in which a tertiary post-treatment was implemented, and the overall energy requirements for treating sewage in such WWTPs could be as low as 0.65-0.70 kWh/m^3 (Iglesias et al., 2017). Membrane fouling is one of the most important drawbacks of the MBR technology. This phenomenon has been defined as the deposition of inorganic and organic substances either on the membrane surface or in the pores of the membrane (Drews, 2010). However, it has not been accurately described yet since the severe complexity and the interactions of the involved factors which impact in declining the capacity of the membrane. Both energy consumption and fouling are related, since an important share of the membrane aeration demands is driven to prevent the cake layer formation, increasing energy requirements (Brik et al., 2006; Drews, 2010). Additionally, fouling has been associated with a detrimental hydraulic capacity of the MBR system.

In the last decades, biofilm processes have been launched as an integrated solution for increasing the capacity of the traditional CAS wastewater systems. These processes are based on the attachment of the microorganisms onto the surface of any support medium for the initiation of a microbial biofilm. Once the biofilm is developed, a self-regulated ecosystem is established (Karadag et al., 2015; Yang et al., 2010). Moreover, the microbial diversity in biofilms could be different to that found in suspension, in those systems in which biofilms and flocs coexist (Nogueira et al., 2002). The use of small suspended biofilm carriers was successfully proven for enhancing organic matter and nutrients removal in conventional systems (Rodríguez-Hernández et al., 2014). These alternatives based on the coexistence of both suspended and attached biomass, have been commonly known as hybrid systems. Thus, this approach should be always considered when studying the possibility of upgrading either urban or industrial WWTP based on CAS processes (Lazarova and Manem, 1995). By adding these carriers and developing a biofilm onto their surface, the biomass concentration can be increased approximately an equivalent of 1.5-2.0 g/L of activated sludge (Metcalf&Eddy et al., 2014). As a consequence, it has been feasible to treat up to twice or three times higher organic and nitrogen loading rates, leading to a space demand much lower than in CAS systems.

In the 2000's, a new approach known as hybrid MBR (HMBR) was released (Garrido et al., 2003). As a result of MBR and biofilm combination, it was foreseen the empowerment of their benefits as treating higher loads of pollutants in less land and the reduction of drawbacks such as membrane fouling. This alternative has been especially appropriate when land scarcity or strict discharge limits are important requests.

A previous study with a lab scale HMBR, achieved nitrogen and organic loading rates of 1.8 kg N/(m³·d), and 6.5 kg COD/(m³·d), respectively. COD removal was 95% and ammonium was fully nitrified, treating industrial wastewaters from a tannery factory (Artiga et al., 2005). COD and TN of the incoming wastewater were 800-1,300 mgCOD/L and 120-160 mgN/L. The reactor was operated at SRT comprehended between 1 and 10 days. In another study, a pilot-scale HMBR fed with fish-canning wastewater, they were achieved an OLR of 4 kg COD/(m³·d) and an NLR of 0.7 kg N/(m³·d). Up to 92% and 95% of COD and nitrogen removal were obtained,

respectively (Artiga et al., 2008). Rodríguez-Hernández et al. (2014) treated municipal wastewater in a HMBR of 1.8 m^3 volume (SRT up to 180 days and HRT 9-12 h), with COD and nitrogen removal rates up to 1.80 and 0.05 kg /(m^3·d). The incoming COD and TKN were 372 and 24 mg/L, respectively. Other study with municipal wastewater also pointed out that the presence of both biofilms and suspended biomass in an HMBR improved nitrogen removal in comparison with a similar MBR containing only biomass in suspension (Liu et al., 2010). In both studies, nitrogen denitrification was restricted to the inner parts of the biofilm.

The impacts of biofilm systems are not only limited to the capacity of upgrading CAS systems, also suspended carriers may influence the membrane performance. Thus, the carrier selection could either increase or decrease the membrane fouling. Kurita et al. (2016) stated that carriers' material had a determinant impact on Fouling Rate (FR). In their work, when rope carriers where added, FR increased. In contrast, if either granular of sponge carriers were present FR diminished. Sánchez et al. (2013) also found that the presence of biofilms diminished the FR, due to the significantly lower amount of colloids detected when biofilms were present in the MBR. Moreover, biofilms provided anoxic zones in their inner parts, promoting nitrogen removal through the denitrification process (Kurita et al., 2016; Rodríguez-Hernández et al., 2012).

Despite the knowledge achieved in the last decades with regards to membrane technologies, the use of MBRs and especially HMBRs for treating low strength municipal wastewater still remains a challenge. This kind of municipal wastewater is commonly produced in cities with high pluviometry and/or when a combined sewerage collection network is available. Rain and underground water led to a dilution effect of the collected wastewater. Thus, a study on the HMBR applicability seemed to be an adequate option.

The main aim of this study is to present the results obtained in an HMBR operated in a municipal WWTP for the treatment of primary settled low strength and alkalinity municipal wastewater.

3.2. Materials and methods

3.2.1. Experimental setup

The pilot plant used in this study was implemented in a WWTP located in Vigo, NW Spain. This WWTP has a design capacity of 400,000 equivalent inhabitants (PE.). The treated water is discharged in an estuary containing bathing and aquaculture areas. The pilot plant (Figure 3.1 and Figure 3. 2)
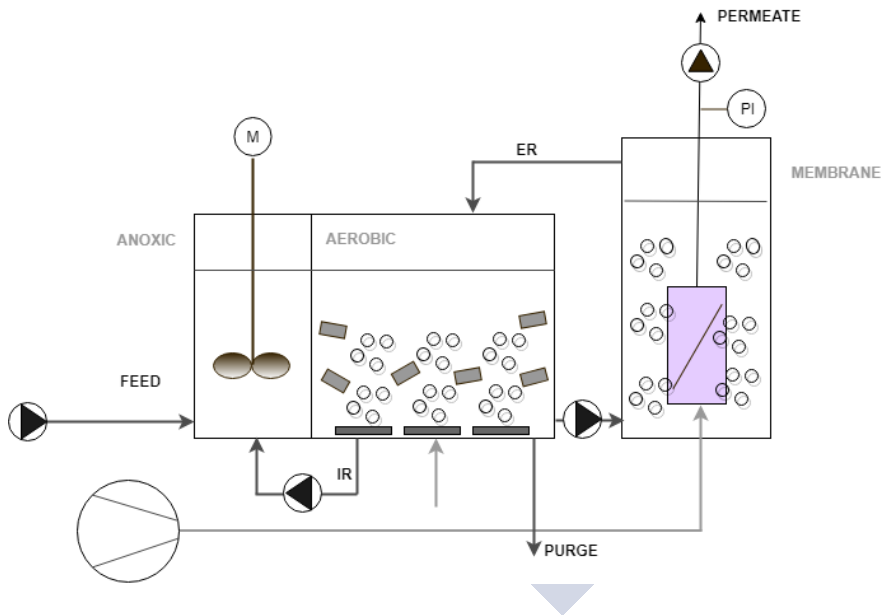
Figure 3.1. Flowchart of the pilot plant HMBR. From the left to the right: anoxic (only suspended biomass), biofilms (with biofilm carriers & suspended biomass) and membrane (with suspended biomass) compartments.
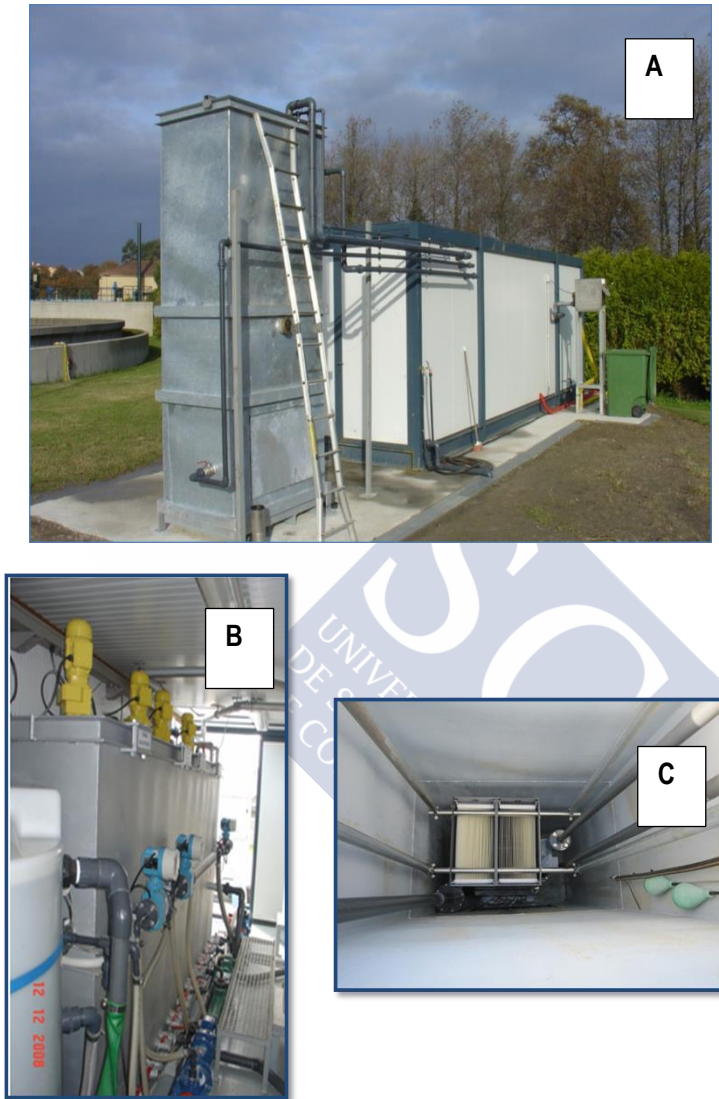
Figure 3. 2 Pictures of the pilot plant. A) External view of the facility. B) Biological anoxic/aerobic hybrid reactor. C) Plan view of the membrane filtration compartment.

The scheme of the HMBR is shown in Figure 3.1. The HMBR, with a total capacity of 4.4 m^3, consisted of three compartments. In the first, a stirred anoxic reactor of 0.9 m^3 volume, named as anoxic compartment hereafter, only suspended biomass was used. The second, of 1.7 m^3 volume, contained both biofilms attached onto carriers and suspended biomass. Both, suspended biomass and biofilms were maintained in suspension by aeration. This compartment, named biofilm compartment hereafter, was filled with 40% v/v biofilm carriers (BioWater®, BWTX, Norway) to enhance the growth of biofilms (more technical data included in Table 3. 1). The third compartment of 1.8 m^3, named membrane compartment, only contained biomass in suspension. An ultrafiltration submerged flat sheet membrane module Microdyn Nadir Bio-Cel® BC50F-C25-UP150 was used during the experiments (more technical data included in Table 3. 2). The effective surface area was 50 m^2 and the pore size of 0.04 μm. The membrane was operated with an specific aeration demand (SAD_m) of 0.7 $Nm^3/(m^2 \cdot h)$, as recommended by the manufacturer. The filtration compartment incorporated an innovative membrane mechanical cleaning system. It consisted of the scouring of the membrane surface by means of the fluidization of 3 mm polypropylene granulate particles (Siembida et al., 2010), which were fluidized by the aeration. Due to the space constraints, the two first compartments of the HMBR (anoxic and biofilm compartments) were located inside a container and the membrane filtration compartment was located outdoors. Additionally, proper screens were located in the biofilm and membrane compartments to retain either the biofilm carriers or granulate membrane cleaning particles, in their respective compartments.

Table 3. 1. Technical data of biomass carriers Biowater Technology BWTX™ (Norway) (left). Image of the colonized biomass carriers (right). Source: Biowater Technology.

Parameter	Description
Width/height (mm)	15 (± 0.5)
Length (mm)	9 (+ 0.2 / 0.5)
Perimeter outside (mm)	56
Perimeter inside (mm)	197
Wall thickness(mm)	0.35 ± 0.1
Density (kg/m^3)	131
Specific surface (m^2/kg)	4.80
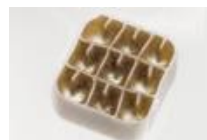
Table 3. 2. Technical data of membrane Microdyn Nadir Biocell® BC50F-C25-UP150 (Germany) (left). Image of the membrane module with the abrasive effect of the granular material (right). Source: Microdyn Nadir.

Parameter	Description
Membrane	
Configuration	Flat sheet
Material	Polyether sulfone
Surface (m^2)	50
Pore size (μm)	0.04
Dimensions (width x length x height) (mm)	702 x 695 x 1563
Operating pressure (mbar)	- 30 – - 400
Backwash pressure (mbar)	max. 150
Operation temperature (ºC)	max 150
Range of pH	2 – 11
SAD_m ($m^3/m^2 \cdot h$)	0.7
Granular material	
Diameter (mm)	2.5
Density (kg/m^3)	1,050
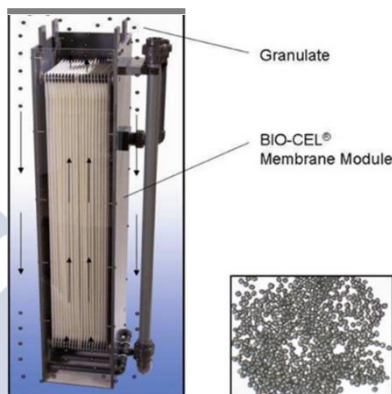
When required, the capacity of the pumps and blowers was modified by means of variable frequency drivers. From day 98 to 105, the oxygen concentration in the biofilm compartment was controlled using an on/off control system of the blower, set to 5 min on - 5 min off. The operation of the system was monitored and regulated by a programmable logic controller (PLC) Allen Bradley MicroLogix ® 1763-L16BWA.

Two recycle streams were included in the HMBR (Figure 3.1). An internal recirculation (IR) stream was addressed from the biofilm to the anoxic compartment to lead drive nitrogen anions to perform the denitrification process. Additionally, an external recirculation (ER) recycled biomass from the membrane to the biofilm compartment. The main operational parameters of the pilot plant are summarized in Table 3. 3.

Table 3. 3. Operational parameters established in the pilot plant.

Parameter	Average	Minimum	Maximum
Incoming flow (L/h)	778 ± 120	514	1,041
IR	1.5 ± 0.4	1.2	2.1
ER	1.3 ± 0.5	0.3	1.9
HRT (h)	5.7 ± 0.8	4.2	8.6
SRT (d)	20	-	-
DO aerobic (mg/L)	1.6 ± 0.9	0.2	4.9
OLR (kgCOD/m^3·d)	0.9 ± 0.2	0.6	1.5
NLR (kgN/m^3·d)	0.14 ± 0.03	0.07	0.24
Membrane flux (L/m^2·h)	-	15	20

3.2.2.   Sampling procedure

Composite samples were taken twice a week from both the feeding and the permeate of the pilot plant, using two autosamplers. These processes consisted of collecting samples every hour in a single basin to make an averaged daily sample. For this purpose, autosamplers Teledyne ISCO 3700 were employed.

Apart from the daily composite samples, grab samples were collected every hour during a day, to determine the pollutants profile variation of the wastewater. Once the samples were collected, each four consecutive bottles were totally mixed and homogenized to diminish the analytical work. These characterization profiles were

carried out four times during the present study, during the operating days 29, 33, 41 and 58.

3.2.3. Analytical methods

Total and soluble chemical oxygen demand (COD_T and COD_S, respectively), ammonium, nitrite, nitrate, total nitrogen and total phosphorus concentration were measured with Hach Lange LCK (Germany) cuvette tests. Biological oxygen demand in five days (BOD_5) was measured with a WTW Oxitop® IS6. Temperature and dissolved oxygen (DO) were measured with a multi-parametric meter Hach HQ40d with the luminescent optical probe InteliCAL LDO101; a portable pH-meter Crison PH-25 was employed. Alkalinity was determined according to the Standard Methods (Rice et al., 2012).

Mixed Liquor Total/Volatile Suspended Solids (MLTSS) were measured as indicated in the Standard Methods (Rice et al., 2012). Colloidal biopolymer clusters (cBPC) were measured accordingly to the procedure described by Sánchez et al. (Sánchez et al., 2013). cBPC have been defined as a pool of colloidal organic matter in the liquid phase of the MBR sludge. It was measured as the difference of the total organic carbon concentration present in a sample of the mixed liquor of the membrane compartment filtered through a 0.45 µm nitrocellulose filter and that measured in the permeate of the membrane with a pore size of 0.04 µm. Transparent exopolymer particles (TEP), have been measured in terms of xanthan glue equivalents, as published in De La Torre et al. (2008).

3.2.4. Nitrification activity assays

Nitrification activities of both suspended biomass (MLSS) and attached biofilm have been measured or estimated by using two different assays. On the one hand, the activity performed by the suspended biomass has been quantified by means of a respirometric assay with a Biological Oxygen Monitor (BOM) device BOM 5300® YellowSpring Instruments (YSI). Sludge sample of MLSS has been taken from the pilot plant and gently washed three times using phosphate buffer solution. The sludge biomass was saturated with oxygen for a period of 12 h. Aeration was disconnected to determine the endogenous respiration by monitoring over time the dissolved oxygen

concentration. Afterward, ammonium was externally injected to assess the ammonium oxidizing capacity by means of the oxygen consumption trends. The actual nitrification capacity was determined as the velocity of the oxygen consumption less the fraction related to the endogenous oxygen demands.

Additionally, biofilm activity has been tracked by means of batch experiments. 25 biomass carriers picked directly from the aerobic compartment were gently washed with phosphate buffer solution (composition in Table 3. 4). The activity assay was run in a 500 mL vessel where phosphate buffer solution, ammonium and sodium bicarbonate were present. Liquid phase samples were taken each 60 min to check the reduction/increase of ammonium, nitrite and nitrate concentrations.

Table 3. 4. Phosphate buffer solution concentration.

Buffer composition	g/L
K_2HPO_4	3,970
KH_2PO_4	3,309
$MgSO_4 \cdot 7H_2O$	1,840
$MgCl_2 \cdot 10H_2O$	1,52
NaCl	0,80
Trace solution	5,00 (mL/L)

3.2.5. Simulation of the experimental results

A simulation software, Biowin® version 5.2, was used to assess the results obtained in the HMBR. The study has been performed with the settled water approach. This part of the research aimed at determining the correspondence between both the experimental data from the demonstrative plant and the predicted values by the simulator under steady state.

3.2.6. Membrane performance

The HMBR was operated with a membrane flux (J) set at either 15 or 20 $L/(m^2 \cdot h)$ during the whole experimental period. The filtration capacity of the membrane has been additionally tracked by typical parameters such as transmembrane pressure (TMP) and permeability.

On the other hand, biopolymer clusters (BPC) were defined as a pool of non-filterable organic matter in liquid phase of the MBR sludge mixture larger than soluble microbial products (SMP) (Sun et al., 2008). Later, Sánchez et al., (2013) assigned the difference in dissolved organic carbon (DOC) concentration between the sludge mixture and after filtration through 0.45 µm filters and the permeate to the colloidal fraction of BPC, known as cBPC. Transparent exopolymer particles (TEP) are organic particles present in sea and freshwaters consisting mainly in polysaccharides which can be observed on the biofilm formed on the membrane (Arruda-Fatibello et al., 2004). Both, cBPC and TEP, can lead to a fouling rate increase. These were measured with the protocols published in Sánchez et al. (2013) and Arruda-Fatibello et al. (2004); respectively and compared with the evolution of membrane fouling.

The critical flux has been well recognised as an adequate tool to assess the actual membrane filtration capacity. It was defined as the flux above which the cake layer formed on the membrane surface by the filtration process is not removable by regular anti-fouling mechanisms such as aeration scouring or backwashing (van der Marel et al., 2009). From a practical point of view, the method employed was the flux-step incremental method proposed by van der Marel et al. (2009). As a standard criterion, the flux is critical flux was attained when the observed TMP declined higher than 10 Pa/min (Le Clech et al., 2003). This assay has been periodically conducted throughout the experimental period.

The resistance to filtration of a sludge sample was determined by a dead-end filterability test. The test was conducted at 25 ºC in an 180 mL pressurized cylinder (Amicon 8200®, Merck Millipore), using a 0.2 µm flat sheet PVDF membrane filters (Durapore®; Merck Millipore). The cell was 100 mbar over pressured by flushing nitrogen gas. When filtration started, soft agitation was switched on and the permeate was measured with time by weighting. The same procedure was accomplished with distilled water, activated sludge and with the colloidal fraction of the activated sludge. The Carman-Kozeny equation has been employed to calculate the cake resistance (m^{-1}). Thus, the pressure drop of the fluid flowing through the sludge cake was measured. Cake resistance was determined by considering laminar flow of the fluid and taking into account that the filtration took place at constant pressure.

3.3. Results and discussion

3.3.1. General results and daily profiles

The fed wastewater was characterised by its low strength with COD_T of 219 ± 38 mg/L, BOD_5 124 ± 33 mg/L, TN 31 ± 6 mg/L TP 4 ± 1 mg/L and a low alkalinity of 302 ± 52 $mgCaCO_3$/L. The average temperature was 18 ºC and the turbidity always below 88 NTU. The average COD_T/TN was of 7.1. The permeate average composition was COD_T 23 ± 12 mg/L (91% removal), Figure 3.3, BOD_5 3 ± 2 mg/L (98% elimination), TN 17 ± 4 mg/L, Figure 3.4, TP 3 ± 1 mg/L (30% removal) and an exhausted alkalinity of 17 ± 8 $mgCaCO_3$/L (95% depletion). Details about influent and effluent characterizations are summarized in Table 3. 5. Turbidity values were always below 0.9 NTU (99%). Observed biomass yield (Y_{obs}) was between 0.22 and 0.25 kgVSS/kgCOD, lower than other reported values of 0.38 kgVSS/kgCOD in an MBR system treating the same municipal wastewater, operating only with biomass in suspension (Iglesias-Obelleiro et al., 2012). Moreover, influent, effluent characteristics and removal efficiencies were accordingly to those previously reported by Iglesias-Obelleiro et al. (2012).
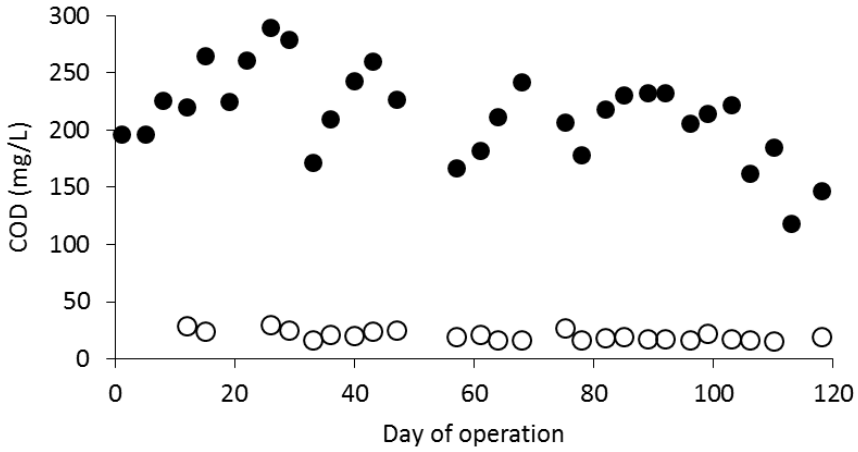
Figure 3.3. Evolution of the COD fed to the HMBR (●) and measured (○) in the permeate.
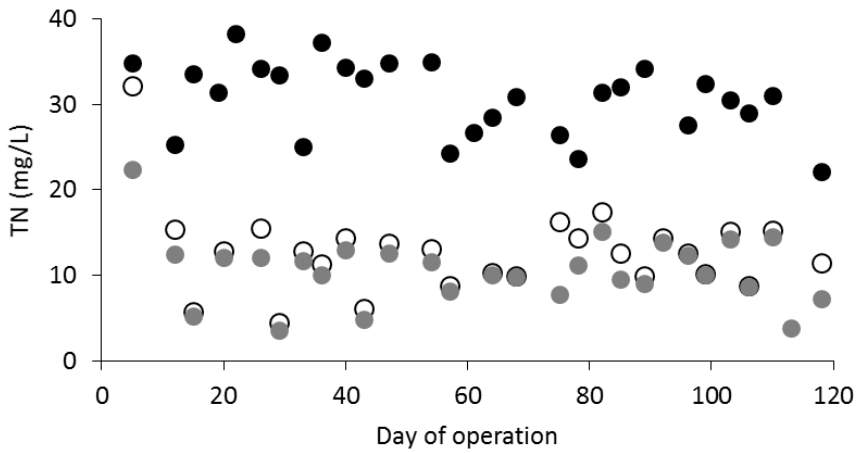
Figure 3.4. Daily evolution of nitrogen. Total Nitrogen fed to the HMBR (●) and found in the permeate: nitrogen anions (●) and sum of nitrogen anions and total nitrogen (○).

Table 3. 5. Wastewater characteristics and removal percentages (average values and standard deviations).

Parameter	Inlet	Outlet	Removal (%)	Number of samples	Biowin simulation	
					Input	Output
COD (mg/L)	219 ± 38	23 ± 12	90 ± 11	31	220	19
Sol. COD (mg/L)	77 ± 16	20 ± 4	74 ± 8	26	-	19
BOD$_5$ (mg/L)	124 ± 33	3 ± 2	98 ± 2	27	-	1
Sol. BOD$_5$ (mg/L)	40 ± 10	3 ± 2	93 ± 3	20	-	1
TSS (mg/L)	78 ± 5	2 ± 1(1)	97 ± 2	26	-	0
VSS (mg/L)	67 ± 7	1 ± 1(1)	98 ± 2	26	-	0
TP (mg/L)	4 ± 1	3 ± 1	30 ± 10	26	4	3
TN (mg/L)	31 ± 6	17 ± 4	49 ± 15	28	31	16
N-NH$_4^+$ (mg/L)	23 ± 5	2 ± 2	94 ± 5	28	-	0
N-NO$_2^-$ (mg/L)	0.1 ± 0.10	0.3 ± 0.1	-	28	-	-
N-NO$_3^-$ (mg/L)	0.5 ± 0.5	10 ± 4	-	28	0	15
pH	7.2 ± 0.3	6.1 ± 0.6	-	28	7.3	-
Alkalinity (mgCaCO$_3$/L)	302 ± 52	17 ± 8	75 ± 5	24	302	-

(1) Both TSS and VSS measured in the permeate were below the detection limit of the method of 10 mg/L.

The HMBR has been operated at a short HRT of 5.7 ± 0.8 h, OLR 0.9 ± 0.2 kgCOD/m^3·d, and NLR 0.10 ± 0.02 kgN/(m^3·d). These values were referred to the whole reactor including anoxic, biofilm and membrane compartments. These values were according to those referred to large-scale MBR facilities treating sewage (Iglesias et al., 2017; Liu et al., 2010). SRT was maintained at 20 d.

Average TN removal was 49%. Taking into account the reported information and the experimental results, COD$_T$/NT, recycling ratio or a combination of both factors could have contributed to the low TN removal percentages. A wastewater with a similar COD$_T$/TN ratio of 6.5 required of an additional electron source (pyrite, Fe$_2$S) for reaching a nitrogen removal percentage up to 95% (Kong et al., 2015). A different configuration of hybrid biofilm/suspended biomass system was tested by Rodríguez-Hernández et al. (2014) with the carriers encapsulated onto a mesh and a recycle ratio set at 3.0. A higher strength wastewater (COD$_T$/NTK of 9.5) was fed. COD, TP and solid elimination percentages were similar to the current study. Nevertheless, a removal TN efficiency of 75% was reported by these authors (Rodríguez-Hernández et al., 2014).

The MBR was simulated using the Biowin software under stationary conditions. Operational parameters (Table 3. 3) and influent composition were considered in a first stage to compare the results from simulation with those obtained experimentally in the permeate (Table 3. 5). Results obtained under stationary conditions, regarding COD, TP, TN and nitrogen ions concentration obtained by simulation were quite similar to those shown in Table 3. 5. For the case of the TP and nitrogen ions concentration the difference between the concentrations experimentally measured or simulated was lower than a few tenths of a milligram per litre.

The daily influent variability has been tracked by means of hourly grab samplings. In Figure 3. 5 an example corresponding to the day 29 has been plotted. A strong variation of some wastewater characteristics has been detected within the day. Remarkable changes in the BOD$_5$/TN ratio were especially noticed. In all analyses, low BOD$_5$/TN ratios were detected. As an example, focusing on BOD$_5$ and TN concentrations, a much higher variability in the influent BOD$_5$ concentration throughout the 24 h profile (BOD$_5^{8:00PM}$/ BOD$_5^{00:00AM}$ ≈ 2.5) could be observed in comparison to the total nitrogen concentration variability (TN$^{4:00PM}$/TN$^{8:00AM}$ ≈ 1.6). This might be due

either to discharges to the sewer system from surrounding factories, generally with lack of nutrients, or by partial organic matter decomposition in the sewer municipal network. Moreover, influent alkalinity of the raw wastewater was extremely low and might be as a result of the geology in this NW area of Spain.
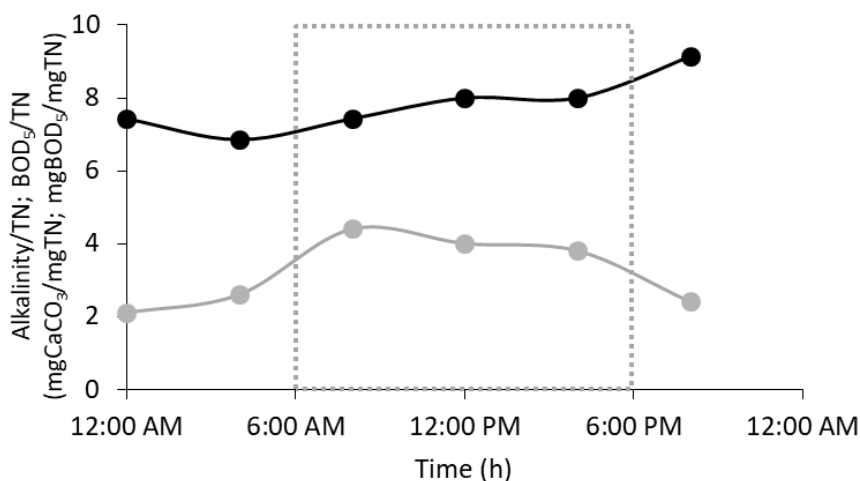

Figure 3. 5. Alkalinity/TN (●) and BOD$_5$/TN (●) daily profile of the influent. The framed area represents the period where denitrification is expected to be maximum.

The estuary where the effluent was discharged was declared as a non-sensitive area. COD$_T$, BOD$_5$ and solids removal percentages fulfilled the EU legal limits Directive 91/271/EC (EEC Council, 1991). Nevertheless, this estuary hosts both bathing and intensive shellfish aquaculture areas. For this reason, microbiological parameters in the effluent should be as low as possible. The Bathing Directive (Directive 2006/7/EC, (EEC Council, 2003)) regulates that *Intestinal Enterococci* should account below 100 cfu/100 mL (Coliform Forming unit) and *Escherichia Coli* up to 250 cfu/100 mL in coastal water for being considered as an excellent quality output. Shellfish Harvesting Directive (Directive 2006/113/EC, (EEC Council, 2006)) states that *Faecal Coliforms* shall stay below 300 cfu/100 mL. Thus, the use of membrane bioreactors is interesting and it has been considered one of the Best Available Technologies (BAT) for providing high-quality standards (Judd, 2008) to the permeate, including microbial indicators. In a previous study by Iglesias-Obelleiro et al. (2012), in presence of a membrane with a

similar pore size than the one employed in the current study, absent values of *Escherichia Coli*, *Total Coliforms*, *Faecal Coliforms* and *Intestinal Enterococci* were registered. Thus, even not being a target of the current study, these results confirmed the fulfillment of all microbiological legislated parameters when using membrane bioreactors.

3.3.2.   Nitrogen removal

Almost all organic and ammonium nitrogen was fully nitrified. Ammonium concentration was 23 ± 5 mgN-NH$_4^+$/L in the influent and 2 ± 2 mgN-NH$_4^+$/L in the permeate. Both biofilm and membrane compartments were aerated and connected through ER stream (Figure 3.1). The average DO concentration in the biofilm compartment was 1.6 ± 1.0 mgO$_2$/L and it was close to the saturation value in the membrane compartment since severe aeration was supplied for preventing the membrane fouling. The nitrogen anions detected in the biofilm compartment were 1 ± 1 mgN/L and 11 ± 4 mgN/L in the permeate, indicating that nitrification took place majorly in the membrane compartment. This fact will be further discussed by means of discontinuous nitrification activity tests.

Nitrogen removal was fairly limited to around 49%. Denitrification took place in the anoxic compartment. ORP was maintained between -20 and -243 mV. These values indicated DO depletion and a reductive ambient (Saby et al., 2003) in this compartment. Denitrification was limited due to a double reason. On the one hand, the strong daily variable and low BOD$_5$/TN ratio, which caused large periods of electron donor scarcity. On the other hand, the low IR applied was only 1.6, due to the restricted IR pump capacity. In these conditions, nitrogen removal was limited to 62%, due to the organic matter limitation of the wastewater. The presence of nitrate in the effluent (10 ± 4 mg N/L) denoted that denitrification was restricted, particularly when the BOD$_5$/TN ratio was around 2 or lower, from 6 p.m. to 4:00 a.m. (Figure 3. 5) since electrons to denitrify were not available (Metcalf&Eddy et al., 2014). Nitrification consumes twice the alkalinity than denitrification recovers (Li and Irvin, 2007). Thus, the buffer capacity of the system was exhausted as a consequence of a double reason: 1) overall available alkalinity was not enough for fulfilling the nitrification necessities of the incoming ammonium and 2) alkalinity recovery associated to denitrification did not fully

proceed. This fact, led to severe pH drops down to 4.5 in the membrane compartment which could affect to the biomass/permeate filtration. In a previous study, Iglesias-Obelleiro et al. (2012) investigated a modified UCT-MBR system located in the same WWTP and fed with the same wastewater and nitrogen reduction was substantially lower, down to 33%.

Batch assays with both biofilm and suspended biomass have been conducted for obtaining the nitrifying capacity of both biofilm and suspended biomass. Figure 3. 6 showed the evolution of ammonium and nitrate concentration in the batch assays performed to the attached biomass. Nitrite was always below 0.2 mgN-NO$_2^-$/L. Biofilm activity averaged 399 mgN-NO$_3^-$/m^2·d and suspended biomass 53 mg N-NO$_3^-$/gVSS·d, corresponding to 40% and 60% of the total nitrification capacity, respectively. It indicated that autotrophic populations capable of nitrifying were present in both suspended and attached biomass. Nevertheless, during the continuous operation, a scarce nitrification was detected in the biofilm compartment. It was probably due to the low DO availability 1.6 ± 1.0 mgO$_2$/L which limited the presence of oxygen to the external layers of the biofilms, where heterotrophic microorganisms were typically hosted. In contrast, in the membrane compartment, the membrane aeration was enough to satisfy the oxygen nitrification necessities of the remaining nitrogen ions. Thus, these results demonstrated that nitrification capacity in the HMBR could be increased, if required, by increasing DO in the biofilm compartment.
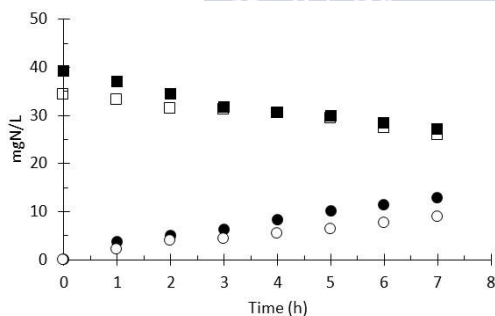
Figure 3. 6. Batch tests performed to determine the ammonium oxidation capacity of the biomass carriers. Ammonium (square) and nitrate (circle) evolution during the experiments carried out during the days 89 (filled) and 122 (white).

The studied HMBR system was proposed as an experimental demonstration for enlarging the capacity of a real WWTP, located in Vigo (NW Spain). The technology focus of this research, offered a compacter alternative in comparison to the installed CAS system. In this way, this based on membrane system demonstrated its capability of treating much larger organic and nitrogen loading rates in the same space occupied by the former Lagares WWTP. A further economic approximation with the experimental data collected in the current study, out of the scope of this work, estimated that an investment of 50 M€ would have been enough to fulfill the wastewater requirements of the upgraded WWTP.

Finally, technicians, engineers and policymakers refuse the idea of enlarging the facility by means of a MBR technology and decided to build a new one in the same grounds. The selected technology was a Veolia's product based on submerged biofilms. An investment larger than 175 M€ was needed to give birth to the new Lagares WWTP, currently in operation. The investment was co-funded by the Spanish Ministry of Environment, the Autonomous Galician Government and the Municipality of Vigo.

3.3.3. Membrane performance

The HMBR was inoculated with activated sludge from the biological treatment of the full-scale WWTP. The initial membrane permeability was 226 L/(m^2·h·bar) when a flux of 15 L/(m^2·h) was set. The permeability ranged from 140 to 291 L/(m^2·h·bar), with an average value of 201 ± 46 L/(m^2·h·bar). TMP ranged between 51 and 151 mbar. The average fouling rate (FR) was 0.95 mbar/d when the flux was set at 20 L/(m^2·h), during most of the experimental period. No chemical cleanings were performed during the whole experimental operation.

Figure 3.7 shows the temporary evolution of the permeability during the experiments. This evolution has been divided into four different periods. In the period I, flux 15 was fixed at 15 L/(m^2·h) (Table 3. 3). MLTSS increased with time and permeability was maintained in the high range, between 220 and 300 L/(m^2·h·bar). MLTSS increased from 1.5 to 4 g/L and this had a positive impact on permeability.
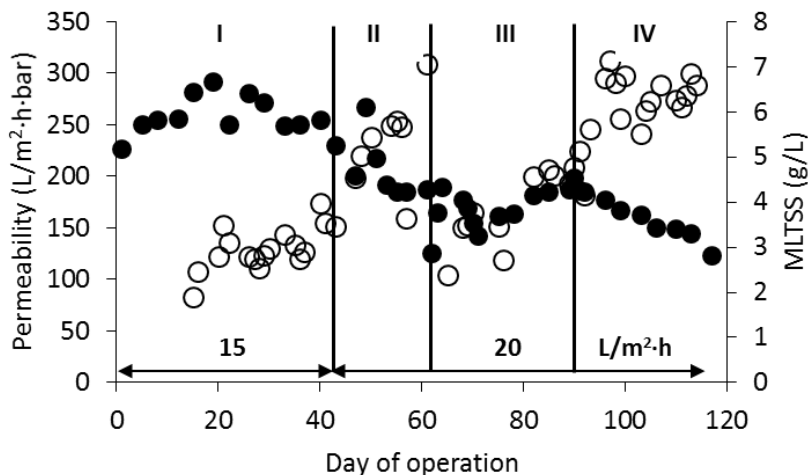
Figure 3.7. Behavior of membrane parameters during the experimental period. In main axis, permeability (corrected to 20ºC) was plotted (●). In secondary axis, evolution of the mixed liquor total (○) suspended solids concentration in the membrane compartment. Both established membrane flux set points were specified.

In period II, Figure 3.7, the performance was different. A decrease in the permeability was noticed and sustained over time. Despite the MLTSS concentration grew, the permeability dropped from 250 to around 200 L/(m²·h·bar). The maximum MLTSS values achieved in this period, 7.2 and 4.3 g/L, were registered in the filtration and biological compartments, respectively. The typical concentrations of MLTSS for conventional MBR systems, 8-12 g/L (Judd, 2011), were never achieved due to the low biomass yield observed, 0.22-0.25 kgVSS/kgCOD, the applied SRT and the punctual losses of biomass caused by sludge overflow. From period II onwards, flux was increased to 20 L/(m²·h). This rise might have caused a negative effect on the permeability of the membrane.

A dramatic drop in the MLTSS concentration happened on the day 61 (Figure 3.7) (beginning of period III) due to a blockage in the filter of the membrane compartment which promoted a massive biomass loss due to overflow. MLTSS concentration dropped down to 3.2 and 1.5 g/L in the filtration and biofilm compartments, respectively. This event caused a severe permeability drop down to 140 L/(m²·h·bar).

When the MLTSS concentration reached a threshold above 4 gMLTSS/L the permeability increased from 140 to 210 L/(m^2·h·bar), indicating that MLTSS had a major impact in permeability evolution. In period IV, a gradual permeability decrease occurred despite a continuous MLTSS gradual increase up to 8 gMLTSS/L, meaning that not only MLTSS and flux had an impact, also more parameters played its role in the membrane fouling.

In periods II, III and IV, when the flux was set at 20 L/m^2·h, the overall averaged FR was 1.2 mbar/d. Available information regarding FR in HMBR operating with municipal wastewater is still scarce. Liu et al. (Liu et al., 2010) performed the experiments with medium strength wastewater. The HMBR was divided into two aerobic compartments. The first aerobic compartment contained Kaldnes K-3 (AnoxKaldnes) carriers. In the second, a hollow fiber microfiltration (0.2 µm) membrane was held. The averaged FR was either 2.80 or 0.86 mbar/d, in absence or presence of carriers, respectively. Other publication dealing with medium strength municipal wastewater at demonstrative scale, incorporated a hollow fiber (0.4 µm) membrane (Rodríguez-Hernández et al., 2014). In this case, biomass support media was encapsulated onto a mesh. Measured FR was 2.76 mbar/d, higher than the observed in the present study. Sánchez et al. (Sánchez et al., 2013) studied a combined UASB coupled in series to an MBR as polishing step with a FR ranged between 1 and 29 mbar/d depending on the operational conditions. Thus, the outputs of the current study were in the range of the lowest when compared to these studies.

pH affects the sludge filterability (Sürücü and Çetin, 1989). This parameter influences not only the stability of the biomass also its activity. Physically, the stressed biomass directly impacts on the rheology (Sürücü and Çetin, 1989) and the amount of polymers segregated by the microorganisms (Drews, 2010), measured in terms of cBPC and TEP in the current study.

Figure 3. 8 showed the results of the off-site filterability tests and the pH of the raw sludge samples tested. As a general behavior, it was noticed that the lower pH the higher filtration resistance. The three highest measured cake resistances were observed for samples with pH below 6.0, where the cake resistance dramatically increased up to the maximum achieved of 4.5·10^{11} 1/m. Low pH-values undoubtedly affects the sludge filterability (Çetin and Sürücü, 1989; Sürücü and Çetin, 1989).
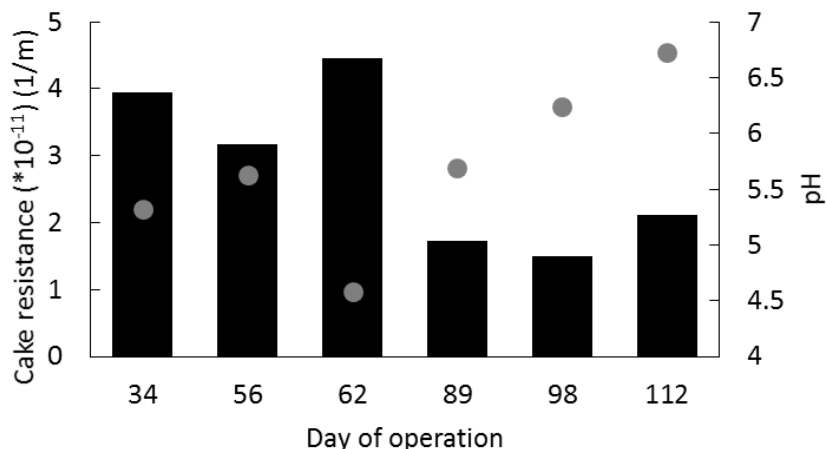
Figure 3. 8. Cake resistance (bar chart) and pH (●) in the membrane compartment influence.

Moreover, critical flux determinations were measured. Three tests were carried out before the operating day 62th, where extremely low pH-values were present in the membrane compartment and severe membrane operating conditions were set. The observed critical flux values were all coincident at 29 $L/m^2 \cdot h$ and ranged in the middle of 15-23 $L/m^2 \cdot h$ (Sánchez et al., 2013; Tiranuntakul et al., 2011) and 32 $L/m^2 \cdot h$ (Iglesias-Obelleiro et al., 2012), in comparison with other studies. From day 62 onwards, three new measurements proceeded with the common result of 26 $L/m^2 \cdot h$. The severe operational conditions affected the membrane capacity. It has not been possible to recover the previous values of 29 $L/m^2 \cdot h$ anymore. The effect of pH values as low as 4.5 turned out to cause an irreversible fouling (Drews, 2010) only recoverable by means of an intensive chemical cleaning.

Other fouling indicators such as cBPC and TEP were tracked. Time evolutions were represented in Figure 3. 9 and Figure 3. 10, respectively.
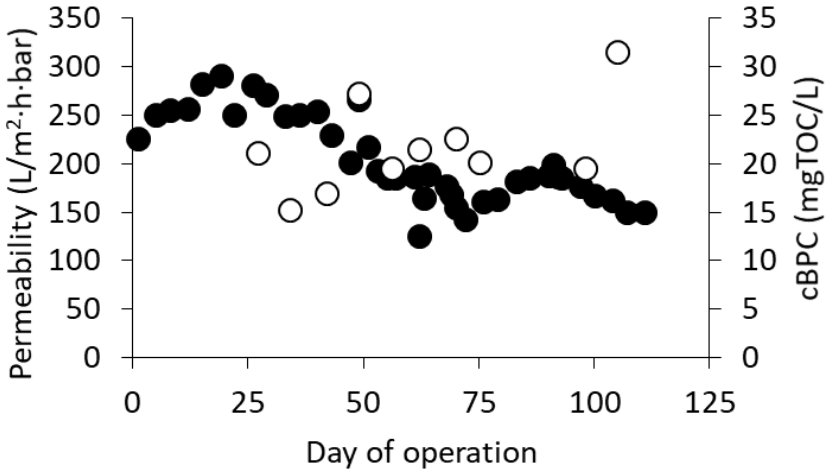
Figure 3. 9. Time evolution of permeability (●) (main axis) and cBPC (○) (secondary axis).
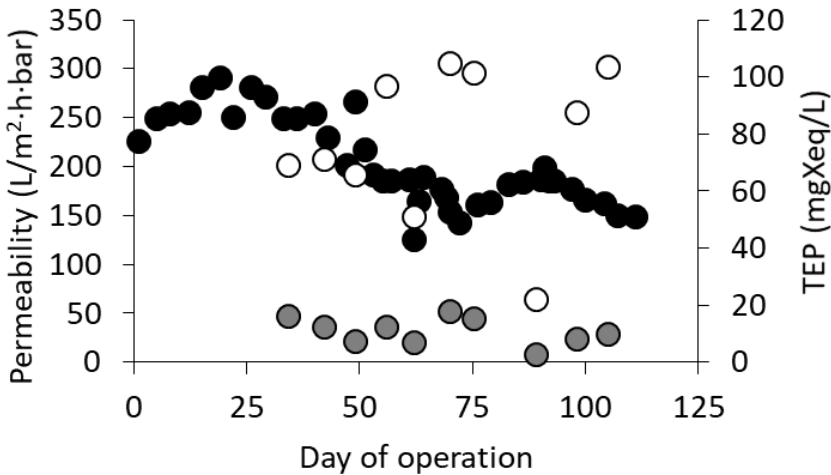
Figure 3. 10. Time evolution of permeability (●) (main axis); TEP in the membrane compartment (○) (secondary axis) and TEP in the effluent (◉) (secondary axis).

cBPC experimental values ranged between 10 and 30 mgTOC/L (Figure 3. 9). These resulted very low in comparison to other works in which this parameter ranged between 10 and 140 mgTOC/L (Sánchez et al., 2013) when feeding a less complex matrix with

a synthetic wastewater. Regarding TEP values, those ranged from 22 to 114 mgXeq/L (Figure 3. 9), which were accordingly to those values included in Sánchez et al. (2013) with values between 10 and 200 mgXeq/L. With these operational values, the correlations published in Sánchez et al. (2013) predicted low FR values confirming the low values experimentally observed.

Figure 3. 11 depicts the experimental correlation between the colloidal fraction of the filtration resistance and the parameters employed for tracking the fouling in this study.
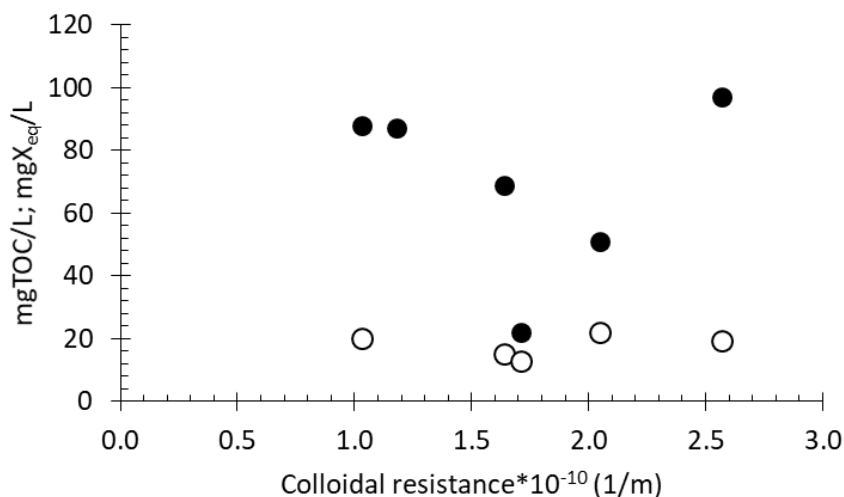
Figure 3. 11. Experimental correlation of the colloidal resistance versus TEP (●) and cBPC (○).

It was observed no correlation between the colloidal resistance and TEP. Similarly, cBPC and colloidal resistance were independent in the range of concentrations involved in this study.

Accordingly to other researches (Drews, 2010), fouling was the result of the contribution of a wide spectrum of parameters and was considered an extremely complex phenomena, with many conditionings involved. TEP and cBPC did not follow a similar trends (Figure 3. 9 and Figure 3. 10), coincidently with other researches. Although many indicators were developed to describe and predict its behavior,

contradictory patterns for the same fact were observed depending on the selected fouling indicator (Wu and Fane, 2012).

Fouling rate and permeability have been accurately calculated in Table 3. 6 and observed FR has been in the range of the lowest in comparison with published results. A strategy for mitigating the fouling with flat sheet membranes has been introduced by Siembida et al. (2010). It consisted of incorporating plastic granules fluidized by means of fine bubbles to scour the membrane surface. This strategy could increase the membrane flux up to 20%. These plastic granules were also used in the current research. The observed permeability values determined in the current study ranged from 140 to 291 L/m^2 h bar, increasing the 100-120 L/(m^2 h bar) stated by Siembida et al. (2010) when operating with municipal wastewater and flat sheet Microdyn-Nadir membranes and mechanical cleaning material. Other MBR configurations treating municipal wastewater (Rodríguez-Hernández et al., 2014) have obtained permeabilities in the same order of magnitude, an average of 210 L/(m^2 h bar). Thus, it can be concluded that the membrane applied in the HMBR pilot plant has shown a promising performance even though significant improvements can be proposed.

Table 3. 6. Behavior of the membrane performance during the experimental periods.

Period	I	II	III	IV
Flux L/(m^2·h)	15	20	20	20
Permeability L/(m^2·h·bar) Initial/final	226/255 (increase)	255/187 (decrease)	187/190 (remain)	190/124 (decrease)
(*) Fouling rate (mbar/d)	-0.16	+1.58	-0.06	+2.24

(*) Negative values of fouling rate means a permeability recovery.

3.4.   Conclusions

The innovative HMBR treating a low strength and alkalinity wastewater has been capable of stably removing more than 90% of COD and 100% of TSS. TN was fairly limited to 49%.

Continuous operation and batch tests demonstrated the reactor's capacity for achieving complete nitrification.

Limited denitrification was due to:

> The hydraulic limitations of the pumping system,
>
> a low COD/TN ratio (lack of electron donors) which in turn provoked
>
> an alkalinity exhaustion.

Membrane performance showed remarkable efficiency indicators: high permeability and a low fouling rate. Fouling indicators (cBPC and TEP) showed low values.

Steady state behaviour can be accurately predicted by the simulator Biowin.

The proposed HMBR alternative seemed to be a good option for enlarging Vigo's WWTP. Nonetheless, further studies are needed for demonstrating an increase in nitrogen denitrification efficiencies.

Chapter 4

Assessment of a combined UASB and MBR process treating wastewater from a seafood industry at different temperatures

Summary

A combined UASB and MBR bench scale system was studied for treating wastewater generated in a food industry. Despite a strong variability in the wastewater characteristics, the system was able to remove the organic carbon compounds and solids. Treated water COD characteristics did not vary and it was lower than the local legal discharge limits within the experimental period. The effect of temperature in the anaerobic UASB stage has been evaluated and organic removal rates (ORR) up to 6 kgCOD/(m^3·d) and COD methanation percentages larger than 90% were obtained. The presence of quaternary ammonium compounds (QAC), used as a biocide for cleaning in the production line, inhibited biological processes, especially anaerobic methanogenic stage and nitrification.

4.1. Introduction

Food, beverage and milk industries employed around 20 million people in rural and industrialized areas of the European Union in 2006 (European Commission, 2006). In the last decades, one of the main concerns of policymakers in this field has been the increase of competitiveness related to these sectors in which an added value higher than 500 MM€ was yearly gained.

Aiming at improving the efficiency in these productive processes, the European Commission has proposed strategies for reducing both the environmental impacts and the costs throughout this sector. Aligned to these goals, schemes focused on reducing the energy consumption and improving the performance of the water cycle, in which the water reuse could be maximized, have been proposed. Wastewater treatment of the generated streams in the productive processes put an eye on these two aspects.

The wastewater (WW) generation in these productive processes may vary from 2 to 40 L WW/ kg of the obtained product (European Commission, 2006), depending on the good manufactured and the efficiency of the process management. To date, industrial WWTP (iWWTP) installed in these facilities are mostly based on conventional activated sludge (CAS) systems, as a result of the large knowledge available. The quality of the treated effluents in CAS systems has typically been a common concern. Thus, the wastewater should be post-treated, in order to obtain a quality which allows to a water reuse in the same factory. Additionally, CAS presents another disadvantage, larger area requirements than other systems such as methanogenic bioreactors or MBR technologies. This situation has been a critical issue in many factories, in which the enlargement of the productive capacity increased the amount of wastewater to be treated. In some cases, the expansion has not been feasible since land scarcity represented a bottleneck, limiting the use of CAS as wastewater treatment system. Thus, in the last years, both MBR and anaerobic treatments, which treated larger organic loading rates, have been explored as alternatives (Iglesias et al., 2017; Lier, 2015). Nevertheless, the main costs associated with these treatments are energy demand, sludge management and chemicals consumption (Iglesias et al., 2017) are influencing operating items which are potentially diminishable if new approaches are installed.

Anaerobic treatments degrade organic pollution, in total absence of oxygen, into methane-rich biogas. This fact entails a direct impact on the energetic demands, the lack of aeration strongly diminished the energetic expenses of the anaerobic treatment in comparison to CAS systems. Furthermore, the obtained biogas can be profitable for energetic purposes. The conversion of this renewable fuel into electricity or heat are common applications of this resource internally in many factories. The obtained biogas diminish the external demands of natural gas, decreasing the operating expenses. Electric efficiencies up to 40% have been observed when working with modern combined heat and power (CHP) gas engines (Lier, 2015). If biogas is directly converted into heat, the energetic performance can account up to 83% (Hakawati et al., 2017). Moreover, sludge production in anaerobic systems has been much lower than the yields generated in aerobic processes (Metcalf&Eddy et al., 2014), impacting on the sludge management costs. Nevertheless, anaerobic treatment only focuses on the COD elimination. The quality of the effluent is lower since solids and nutrients are not removed (Lier et al., 2008). Over 2,200 anaerobic treatment references were installed worldwide between 1981 and 2007 (Lier, 2015) for treating industrial wastewaters. Among them, UASB reactors led the marked in this period with over 50% of the installed references.

MBRs completely retain suspended solids in the system since the pore size is lower than that of solids (Drews, 2010; Judd, 2016). Moreover, nitrogen removal is possible with some configurations of these systems (Buntner et al., 2013; Silva-Teira et al., 2018). For these reasons, remarkable high-quality effluents have been commonly obtained, and even MBR permeate can be reused in the same factory, closing the water cycle. Possible purposes of this recycled water can be in auxiliary applications such as vehicles rinsing/washing and process water for cooling towers and evaporative condensers. Nevertheless, due to the legal and sanitary constraints, the use of reclaimed water is totally prohibited, in those applications in which treated water and edible goods are in contact, such as the main productive process. This strategy was addressed to diminish the water demands of the overall productive process. On the other hand, aeration and sludge production in MBRs are much larger than those respect to anaerobic systems. MBRs market grew in the last two decades (Iglesias et al., 2017; Judd, 2016) and nowadays could be considered a mature technology.

Besides, one of the main drawbacks of methanogenic UASB systems has been the presence of solids, pathogens, organic carbon and nutrients in the treated effluent. In this sense, coupling in series to the anaerobic reactor an aerobic MBR system, as a post-treatment, addressed to polish the remaining organic carbon and the contained solids, was considered a good strategy to obtain an effluent with a reuse quality. Moreover, this in series MBR guarantees the total retention of the biomass in the eventual case of a massive washout from the UASB. A patent of the University of Santiago de Compostela (ES2385002B2) includes both a UASB as methanogenic reactor and an in series 2-stages MBR as polishing treatment. In this way, the patented system aimed at integrating the advantages of both, anaerobic and aerobic systems, improving the quality of the anaerobically treated water and minimizing the energy required for the wastewater treatment. In the first stage, most of the organic carbon is converted to methane-rich biogas. In the second, combining flocculent and biofilm biomass, aerobic treatment is conducted to oxidize the remaining organic carbon and to convert the ammonium, coming from the anaerobic treatment, into nitrate. Additionally, in the first stage of the MBR, an improvement of the flux through the membranes was expected by adding carriers, onto which biofilm was developed. In this, filtration specialized microorganisms were promoted. Buntner et al. (2013) already assessed this system at bench-scale with dairy wastewater with promising results. In this previous study, temperature was maintained in the range of 17-25 ºC and the methanation capacity averaged in 70%. This anaerobic system was fed at an Organic Loading Rate (OLR) ranged between 0.5 and 5 kgCOD/(m^3·d) (Buntner et al., 2013).

In this study, a combined UASB and MBR process which joined anaerobic and MBR advantages has been launched for treating food processing wastewaters. The main aim of this study was to demonstrate the feasibility of the combined UASB and MBR for treating wastewater from a seafood processing factory. In the first UASB reactor, the methanogenic stage was assessed and a large fraction of the organic carbon was expectably removed. The UASB effluent was led to a second stage, a polishing aerobic MBR, in which a high quality effluent expectably reusable was obtained.

4.2. Materials and methods

4.2.1. Experimental setup

The study was carried out in a seafood processing factory located in Galicia (NW Spain). Squid, sole, cod or hake are raw materials employed in this facility.

A schematic of the combined UASB and MBR bench-scale system is shown in Figure 4. 2. The methanogenic treatment was conducted in a UASB reactor of 120 L. This system was provided of an external heating jacket for accurately controlling the reactor's temperature. The MBR (56 L) was composed of two compartments. The first compartment (36 L) was stirred by aeration and is denoted as the biofilm compartment hereafter. In this compartment, biomass was present both in suspension and adhered onto carriers. A mixture of 7 L (apparent volume) of foam (Levapor biocarrier; Levapor GmbH, Germany) and 2 L (apparent volume) of rigid carriers (Mutag Biochip; MultiUmwelttechnologie AG, Germany) were used. Both carriers occupied 26% of the apparent volume of the aerobic compartment.
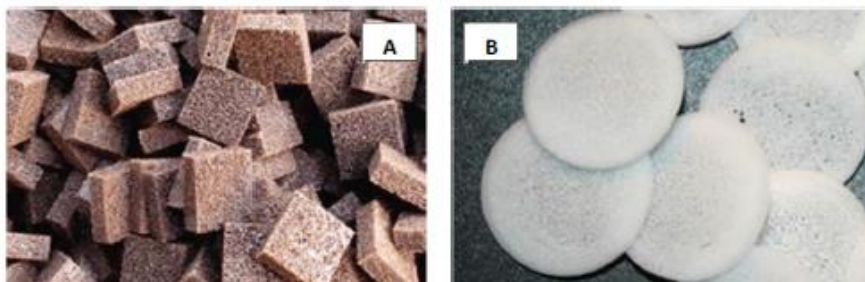
Figure 4. 1. Biomass carriers embedded in the MBR post-treatment. A) Levapor biocarrier. B) Mutag Biochip.

The second, aerated compartment (20 L), known as the membrane filtration compartment hereafter, contained an ultrafiltration hollow fiber membrane (Zenon ZW-10) with an effective filtration surface of 0.9 m^2. The physical separation between the mixed liquor and permeate was achieved using a membrane, with a pore diameter of 0.04 μm. The permeation cycle lasted 7.5 min, including 0.5 min of backwashing and 7 min of filtration. This compartment was aerated with a specific air demand (SAD_m) of 0.7 $m^3/(m^2 \cdot h)$ to minimize membrane fouling. The operation of the system was

monitored by a PLC (Allen Brandley Micrologix 1400) connected to a computer. Transmembrane pressure (TMP) data was measured with an analog pressure sensor (IFM Efector 500 PN 2009) and collected in the PC by an analog Micrologix PLC module.
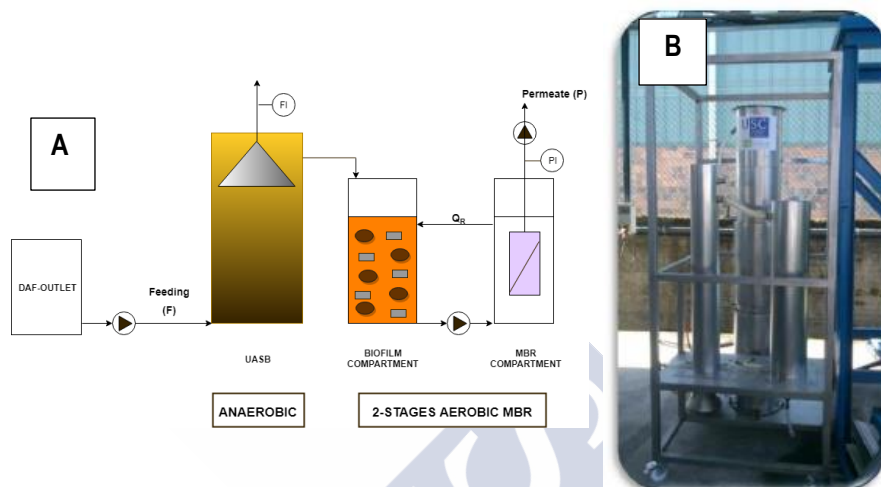
Figure 4. 2. Schematic diagram of the 3-stages UASB + MBR composed by an anaerobic stage (UASB) and an aerobic MBR consisting of a biofilm compartment and an MBR compartment where the membrane was located. B) Picture of the bench scale plant.

For shortening the start-up period, UASB was seeded with granular biomass collected in a brewery factory and the MBR with screened activated sludge from a secondary treatment of a municipal WWTP.

An internal recycle stream (R) from the membrane filtration to the biofilm compartments of the MBR was set up. R-value has been fixed at 2.2 within the experiments.

The system was punctually purged when the Total Suspended Solids (TSS) content increased above 8 gTSS /L, as recommended by Buntner et al. (2013). The mass of TSS withdrawn was measured to estimate the overall biomass yield (Y_{OBS}).

The characteristics of the wastewater generated and fed to the experimental setup are shown in Table 4. 1.

Table 4. 1. Seafood wastewater characterization. SD: standard deviation.

Parameter	Average ± SD	Minimum	Maximum
COD_T (mg/L)	1514 ± 668	308	3008
COD_S (mg/L)	1157 ± 455	280	2156
TN (mg/L)	50 ± 23	12	99
TP (mg/L)	3 ± 2	1	13
TSS (mg/L)	215 ± 198	20	560
VSS (mg/L)	174 ± 274	16	448
pH	6.8 ± 0.9	4.3	8.6
Conductivity (mS/cm)	1.80 ± 0.38	1.21	2.32

4.2.2. Operational strategy

The system was operated for 305 days. Four different operating stages (Table 4. 2) are distinguished according to the temperature at which the UASB was maintained. During a first stage, the temperature in the UASB reactor was initially not controlled (stage I) and operated in the range of 25 to 35 ºC in the remaining stages (from stage II to IV). The MBR was operated at ambient temperature during the whole experimental period. Two different substrates were fed depending on the collection point of the current WWTP: 1) Raw wastewater: before the train of dissolved air flotation (DAF) vessels; 2) degreased wastewater: after the train of DAF vessels.

Table 4. 2 summarized the strategy of the stages indicating the operational temperature of operation of the UASB system.

Table 4. 2. Operational strategy in the UASB system.

Stage	Days of operation	Temperature UASB (ºC)	Substrate (days)
I (Ambient)	0-131	17-25	Degreased
II	132-201	25	Degreased
III	202-254	30	Degreased (202-244)/ Raw (245-254)
IV	255-305	35	Raw (255-288)/ Degreased (289-305)

The anaerobic process can lead to a pH drop of the wastewater due to the low alkalinity of the used wastewater. Thus, industrial grade $Mg(OH)_2$ product with 80% of $Mg(OH)_2$ (Magnesitas de Rubián S.A., Spain) has been punctually added in the feed tank to increase the buffer capacity of the wastewater.

4.2.3.   Analytical methods

Volatile Suspended Solids (VSS), total and soluble Chemical Oxygen Demand (COD), nitrite, nitrate, and ammonium were determined according to the Standard Methods (Rice et al., 2012). Temperature and dissolved oxygen (DO) were measured with a multi-parameter meter with a luminescent optical probe (Hach HQ40d IntelliCAL LDO101). A portable pH meter (Crison PH-25) was employed. Biogas production was measured by using a Milli GasCounter MGC-10 (Ritter, Germany) and its composition was measured in a gas chromatograph (HP 5890 Series II) with the column of Porapack Q 80/100 2 m x 1/8" (SUPELCO). The biogas transportation from the factory to the Department of Chemical Engineering was conducted by means of Tedlar® bags.

Volatile Fatty Acids (VFA) acetic, butyric, propionic and valeric were measured by a gas chromatograph (5890A by HP) equipped with a flame ionizer and an automatic injector (7673A by HP). Total organic carbon (TOC) has been determined in a TOC measurer Shimadzu TOC-5000.

Colloidal biopolymer clusters (cBPC) were measured accordingly to the procedure described by Sánchez et al., (2013). cBPC have been defined as a pool of colloidal organic matter in the liquid phase of the MBR sludge. It was measured as the difference of the total organic carbon concentration present in a sample of the mixed liquor of the membrane compartment filtered through a 0.45 μm nitrocellulose filter and that measured in the permeate of the membrane with a pore size of 0.04 μm.

4.2.4.   Nitrification activity tests

Assays to determine the specific nitrification rate of the biofilm compartment and suspended biomass have been performed during the continuous operation. Flocculent and biofilm biomass was directly taken from the pilot plant. Those samples were gently washed with phosphate buffer three times to remove the remaining oxidizable species.

These assays were carried out in similar conditions than those present in the continuous operation for both suspended and attached biomass.

Biomass samples were continuously aerated and ammonium was externally injected to assess the ammonium oxidizing capacity. The activity assay was run in a 500 mL vessel where phosphate buffer, ammonium (25 mgN/L) and sodium bicarbonate (42 mgNaHCO$_3$/L) were present. Liquid phase samples were taken each 45 min to analyze the evolution of ammonium, nitrite and nitrate concentrations. Tests were run at laboratory temperature at 20ºC.

4.2.5. Cake resistance

The resistance to filtration of the membrane filtration sludge was determined by a dead-end filterability test. The test was conducted at 25 ºC in a 180 mL pressurized cylinder (Amicon 8200®, Merck Millipore), using a 0.2 μm flat sheet PVDF membrane filters (Durapore®; Merck Millipore). The cell was 100 mbar over pressured by flushing nitrogen gas. When the filtration was detected, a soft agitation was switched on and the permeate was measured by weighing. The same procedure was accomplished with distilled water, activated sludge and with the colloidal fraction of the activated sludge. The Carman-Kozeny equation has been employed to calculate the cake resistance (m^{-1}). Thus, the pressure drop of the fluid flowing through the sludge cake was measured. Cake resistance was determined by considering the laminar flow of the fluid and taking into account that the filtration took place at constant pressure. Assays were run at laboratory temperature at 20ºC.

4.2.6. Anaerobic biodegradability batch assays

Biodegradability batch tests in anaerobic conditions were performed to determine the organic carbon contained in the wastewater potentially broken down into methane-rich biogas. These tests were performed by employing the protocol published in Angelidaki et al. (1998).

Wastewater from the current industrial WWTP was used as substrate. Two different samples were taken as substrate, raw and degreased wastewaters. As inoculum, anaerobic granular biomass from a similar setup than the one employed for these

experimental works fed with other substrate was used. Temperature, at 37ºC, and stirring velocity were controlled by an incubator.

4.3.   Results and discussion

4.3.1.   Biodegradability batch tests

Figure 4. 3 represents the evolution of the methane generated, normalized to the mass of inoculum, as a function of time. Each series depicted either raw or degreased wastewater as collection points of substrate in the current WWTP. Figure 4. 4 includes the overall extension of the methanation at the end of the test and two 2 days after the beginning.
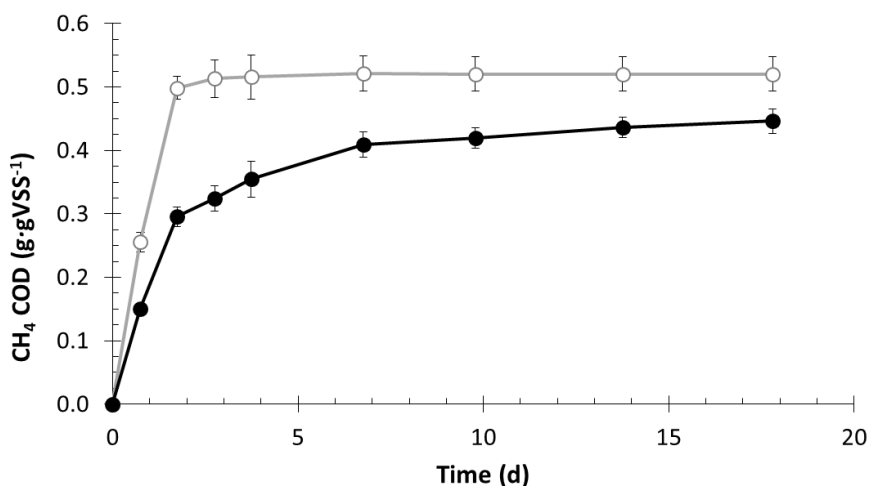
Figure 4. 3. Time evolution for the anaerobic biodegradability tests. Obtained methane per mass of inoculum for the substrates: (○) raw and (●) degreased wastewater.
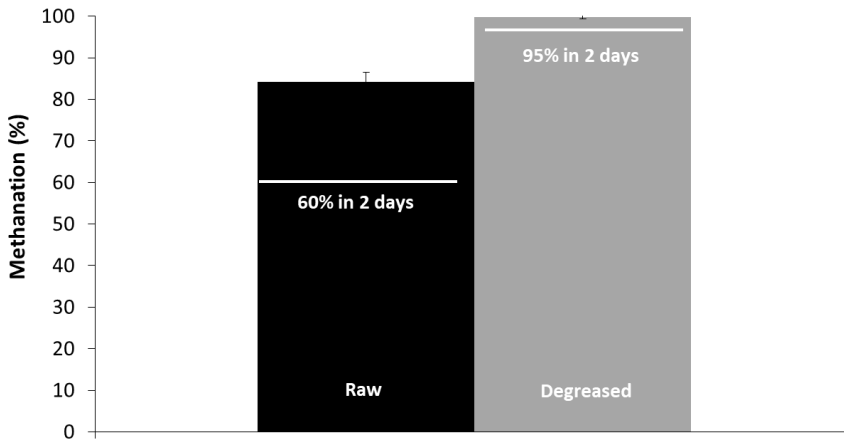
Figure 4. 4. Methanation percentage at the end of the biodegradability test (16 days) and after two days of experimentation. Degreased (○) and raw (●) substrates.

As it can be seen in Figure 4. 3 and Figure 4. 4, both series, corresponding to raw and degreased wastewaters, respectively, behaved in different ways. When the raw substrate was fed, both the biodegradation velocity at the beginning of the test and the overall extension of the obtained methane were lower than in the case of degreased wastewater. In the case of raw substrate, the overall methanation extension accounted up to 85%, being reached a 60% in the first 48 h of assay. When degreased wastewater was the substrate, 98% of carbon was transformed into methane and the 95% was attained in the first two days. The experimented difference in both tests can be probably due to the higher presence of fats in raw in comparison to degrease substrates. The biodegradation complexity increased due to the presence of these large compounds which made the hydrolysis a complex and slow process. On the other hand, those results indicated the high performance of the train of dissolved air flotation vessels related to the separation of the non-biodegradable compounds.

4.3.2.   General results

The system has been operated for 305 experimental days. COD_T, COD_S, TN, and TP in the wastewater fed to the first UASB system were 1514±668, 1157±455, 50±23 and 3±2 mg/L, respectively. pH-value in the influent was maintained in 6.9±0.9. The

average UASB effluent concentrations were COD_T 505±475, COD_S 374±322, $N-NH_4^+$ 45±25 mg/L, and $P-PO_4^{3-}$ 1±1 mg/L.

The anaerobic reactor was operated at the temperatures indicated in Table 4. 2. Measured pH in the UASB outlet averaged 7.3±0.6. HRT, referred to the UASB stage, ranged between 8 and 41 h. The methanogenic treatment led to a COD_T removal of 63±25%, within the experimental stage. Average biogas production was 35-45 L/d with a methane percentage of 71±14%. COD balances in the anaerobic reactor revealed that up to 90% of total COD fed was methanized.

The effluent from the UASB was driven to the aerobic MBR post-treatment system. TSS and VSS ranged between 4-28 and 4-24 g/L, respectively, in the biofilm and membrane filtration compartments. Averaged measured DO and pH in the biofilm compartment were 1.8 mg/L and 8.0±0.4, respectively. The estimated biomass yield, referred to the combined UASB + MBR, was 0.18 $kgVSS/kgCOD_T$, similar to previously reported values (Buntner et al., 2013; Silva-Teira et al., 2017) using the same process and for the treatment of other type of wastewater. In the permeate, COD values were 55±65 mg/L. Regarding TN and TP, their concentrations were 27±25 and 2±2 mg/L, respectively. Suspended solids were totally retained in the MBR due to the membrane's retention capacity. Achieved turbidity values were 1.1±1.1 NTU in the permeate.

With regard to the membrane performance, net flux was maintained at 3-17 $L/(m^2 \cdot h.)$. TMP varied around 13-184 mbar and permeability values ranged between 37-462 $L/(m^2 \cdot h \cdot bar)$.

4.3.3. Impact of temperature on anaerobic stage

Figure 4. 5 depicted the evolution of COD fed, the effluent of the anaerobic UASB and the COD concentration in the permeate. Moreover, the temperature maintained in the anaerobic reactor was also monitored.
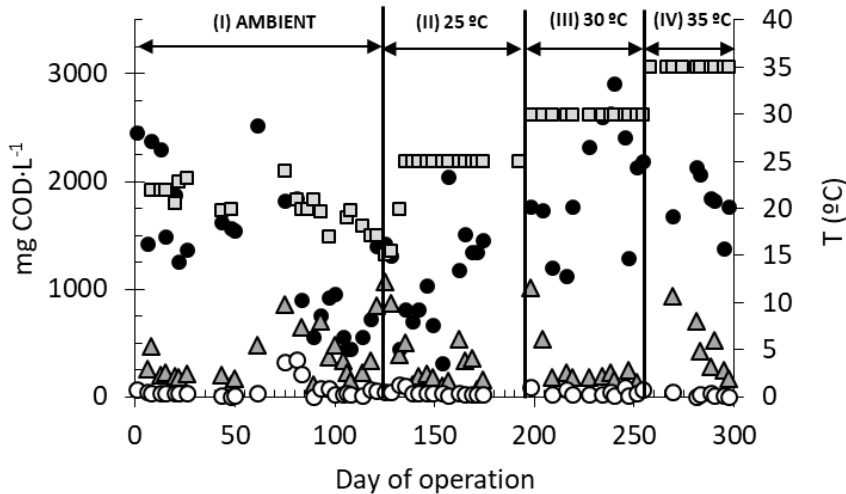
Figure 4. 5. Trends of the organic carbon concentration in the liquid streams. Daily trends of COD_T influent (●); UASB effluent (△) and permeate (○). Secondary Y-axis: daily average temperature (□) in the experimental system. Indicated in the graph is the temperature status in the UASB reactor. Ambient makes reference to the period when the temperature in the anaerobic stage was not controlled.

The main difference between the Stage I (ambient temperature) and the others (stages II, III and IV) was the absence of a temperature control system in the UASB reactor.

Degreased wastewater was fed in Stage I. During the first 61 days, measured temperature varied from 17 to 25 ºC and the anaerobic system yielded as expected (60-80% removal efficiency). From day 61 onwards, significant daily ambient temperature changes were observed which, in turn, directly affected the performance of the anaerobic reactor. Differences between day and night up to 15 ºC were detected in the facility in which the reactor was installed. This fact negatively impacted on the stability of the anaerobic process, since temperature fluctuations typically played a negative role on both microbial interactions and methanation performances of the complex microbiological anaerobic process (Kim and Lee, 2016; Lin et al., 2017). Cha and Noike, (1997) studied the negative effect of rapid temperature changes in acidogenesis. It was found a dramatic decline in the number of acetate-utilizing methanogens, which in turn stopped methanogenesis, when temperature rapidly

decreased in 5 ℃, especially at short HRT 6-12 h (Cha and Noike, 1997). These stated HRT values were in the order of those applied in some periods of Stage I.

During stages II, III a higher degradation performance was observed. A decrease in the COD concentration was observed in the effluent of the UASB, as a consequence of the temperature rise in the methanogenic stage. Once the reactor was adapted to the new controlled temperature, stable COD concentrations in the UASB effluent were reached. COD-values lower than 500 mg/L and below 250 mg/L were attained for stages II and III, respectively. In the last days of period III, raw wastewater was fed without any negative impact in the methanogenic system.

In contraposition, a stable value of the COD effluent was not reached within the Stage IV when raw substrate was fed instead of degreased wastewater into the UASB reactor, even though the 5℃ increase temperature set in the methanogenic reactor (Table 4. 2). It was observed that the rise of temperature set in the methanogenic stage was incapable of facing the complex raw wastewater. These results were according to the biodegradability batch tests previously stated (Figure 4. 3 and Figure 4. 4), which indicated a higher difficulty of anaerobically treating raw than degreased wastewaters. Klaucans and Sams, (2018) stated a similar fact when dealing with wastewater from other food production factory. Co-digestion of the separated fats and oils with primary sludges or scums have been typical strategies followed in other similar WWTPs (Klaucans and Sams, 2018). This was due to an external increase of enzymes specialized in the hydrolysis, present in these primary sludges and scums. Hydrolysis have typically been the bottleneck in the anaerobic process when dealing with wastewaters rich in complex compounds such as fats, greases and oils. The contact of these substances with granular or attached biomass generally led to block the external layer of biofilm. This fact typically impeded a correct mass transfer between the liquid phase and the biomass. In the medium term, this effect normally led to a gradual reduction of the degrading activity and, finally, a general failure of the biological system (Miranda et al., 2005).

During the last days of Stage IV, degreased wastewater fed was restored. As a consequence, a rapid increase in the performance of the methanogenic reactor was recovered. This achievement was in accordance to the anaerobic biodegradability

assays (Figure 4. 3 and Figure 4. 4) which pointed a higher methanation capacity of degreased respect to raw wastewater.
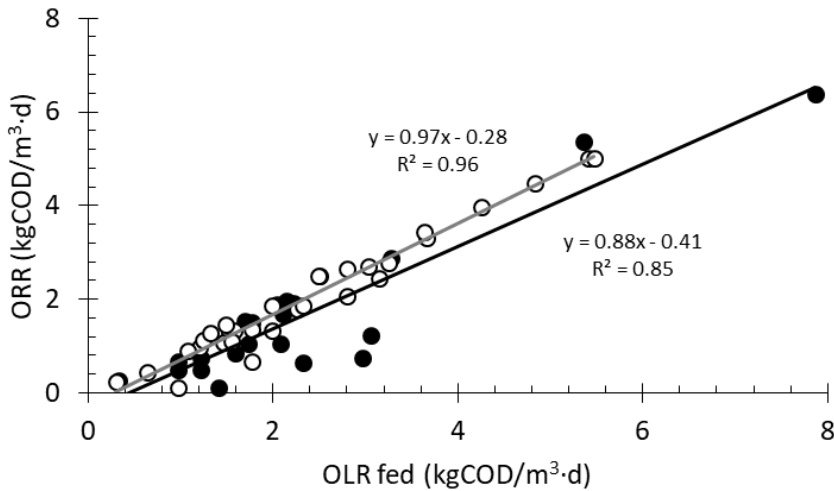

Figure 4. 6. Correlations between the fed organic loading rate (OLR) and the organic removed rate (ORR). Values corresponding to the Stage I were drawn in black (●) and those corresponding to stages II, II and IV were depicted in white (○). Linear correlation for Stage I was shown in black and for stages II, III and IV are depicted in grey.

The slope related to the set of values corresponding to stages II, III and IV was higher than the one corresponding to Stage I. Thus, it was confirmed that the fact of maintaining constant the temperature in the anaerobic stage positively affected the UASB stability (Kim and Lee, 2016; Lin et al., 2017).

Figure 4. 6 represented both fed and removed OLR and volatile fatty acids (VFA) as acidification indicator.
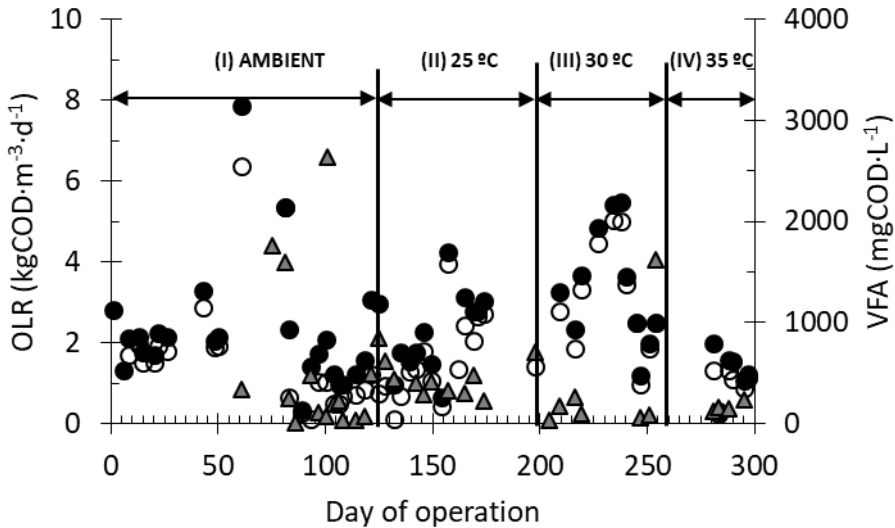
Figure 4. 7. Evolution of the organic loading rate (OLR), all the streams and calculations referred to the UASB volume. Fed to the system (●), removed (○) and VFA (▲) observed in the effluent.

During the first 60 days of operation, the COD removal capacity of the UASB was high, as indicated by Figure 4. 7. Moreover, VFA values in the UASB effluent were always below 200 mgCOD/L, indicating that methanogenesis was not the limiting stage in the overall anaerobic process. Hence, removed OLR up to 3.5 kgCOD/(m³·d) was achieved and the limits of the system were not attained, in line with other researches (Lier et al., 2015).

Nonetheless, COD concentration was only function of the manufacturing product and the type of produced good in the factory. Suddenly, a severe COD increase in the wastewater was noted in the day 61. As OLR was governed by both, inlet flow and the organic carbon concentration in the wastewater, such a rise of COD in the influent entailed fed OLR values as high as 8 kgCOD/(m³·d), out of the advisable ranges for a methanogenic process at 15-25 ºC (Table 4. 2) (Lier et al., 2015). As a consequence, extremely high VFA values (Figure 4. 7) were detected. VFA accumulation indicated that the biological process was stopped in acidogenesis. Methanogenesis was inhibited since methanation percentages were as low as 3% in this period. Once the

fed OLR decreased, the system quickly recovered to negligible VFA values. From day 85, negligible VFA concentrations were detected and COD inlet and removal ranged in expected ranges (Figure 4. 7), indicating that the overload period was overcome.

An intensive cleaning campaign was carried out in the factory during a short period, in which the production was stopped. For performing this tasks, cleaning and sterilizing products were used from the day 93 to 105. Those products included biocides as quaternary ammonium compounds (QAC) (9%) and glutaraldehyde (10%). A fraction of these biocides were present in the wastewater. Consequently, a VFA accumulation event was detected from day 120. QAC have high affinity to adsorb onto biosolids blocking organic carbon/biomass contact. Activity inhibition rates depended not only on QAC concentration also structures, acclimation and the presence of QAC degrading communities played an important role (Tezel et al., 2006). As a consequence, COD elimination was constrained and a relative VFA concentration growth was detected (Figure 4. 7). This fact was also observed in another experimental prototype which was simultaneously running in the same factory and fed with the same wastewater. Both of them, as well as the current industrial WWTP, had been inhibited at the same time. Tezel et al. (Tezel et al., 2006) deeply studied the effect of QAC in a methanogenic batch reactor. Anaerobic process stopped in acidogenesis and a VFA accumulation when a threshold of 30 mg/L was reached. Accordingly, it can be estimated that the combined UASB and MBR system might have been fed with a minimum concentration of 30 mg QAC/L. Once the presence of QAC was cut off, methanogens restored their activity 57 days later (Tezel et al., 2006). This fact indicated that a relatively long term was necessary for overcoming the transient state caused by the presence of QAC to the methanogens. Hence, the inhibitory event observed from the day 120 to 170 was probably due to the presence of a large amount these biocides in the wastewater.

From day 128 onwards, VFA concentrations started to decrease (Figure 4. 7) and COD concentrations in the effluent were below 550 mg/L (Figure 4. 5). Additionally, the fact of setting the temperature controlled at 25 °C enhanced a shortage of the time required for recovering all the way the anaerobic process including methanogenesis. Once the system was restored, the temperature assessment on the anaerobic treatment continued. Buntner et al., (2013) operated the same experimental setup treating dairy wastewater with a slow changeable temperature of 17-24 °C within 292 days,

comparable temperatures to those included in Stage I. Despite this more favorable circumstance and the constant characteristics of the feed, values up to 4.2 kgCOD/(m³·d) were eliminated. In the current experimental setup, similar removal rates have been observed in the ambient temperature stage even operating at less favorable conditions such as more changeable wastewater characteristics and with the temperature daily changes registered. van Lier et al. (Lier et al., 2008) included a summary with the expected values of removed OLR when employing UASB reactors at 25 ℃ with wastewaters not including VFA with similar values ranged between 4 and 8 kgCOD/(m³·d). A comparison between the observed values during the first stage of the current study and those available in the bibliography indicated that the collected values in this research were in accordance to those published. Ahn and Forster (Ahn and Forster, 2002) studied the effect of temperature disturbances. A loss in the anaerobic reactor performance and a reduction in the effluent quality was noted when increasing or decreasing this parameter. This fact indicated that the stability of the temperature in an anaerobic system has been crucial for maximizing its performance, accordingly to the observed during the current study. Thus, a deep study of the anaerobic process was carried out once a temperature control system was installed.

Stages II, III and IV have been characterized by temperature control in the anaerobic UASB at 25, 30 and 35 ℃, respectively. Within the experiments, COD values below 250 mgCOD/L were observed in all samples, considering both the anaerobic methanation and the aerobic oxidation in the post-treatment. As a consequence, a removed OLR up to 5.1 kgCOD/(m³·d) without detecting any acidification indicator and stable methanation percentages between 80 and 91% have been collected. The only sample where VFA values were noticeable was as a result of a temperature drop due to a heating jacket malfunctioning. Accordingly, Ahn and Forster (Ahn and Forster, 2002) observed a sudden increase of VFA, especially acetate, once the temperature of the lab-scale digester rapidly switch from stable 35 ℃ to no-controlled temperature. This study also stated transient declines in methane production also observed when temperature changed. van Lier et al. (Lier et al., 2008) reported removed OLR in this conditions up to 18 kgCOD/(m³·d) when feeding VFA-rich wastewaters at 30 ℃ or, alternatively, when the UASB was set at 35 ℃ fed with absence of VFA in the substrate. In this case, the large methanation percentages and such low COD concentrations in the UASB effluent indicated that the achieved removed OLR values

were not limited by the capacity of the reactor. Moreover, in this stages, VFA values
were negligible. COD concentration of the inlet wastewater and membrane filtration
capacity have been the limiting conditions.

Anaerobically treated effluents in a UASB had low quality due to the presence of high
suspended solids and a remaining COD fraction still high (Lier, 2015). Spanish law
5/2002, about industrial wastewater discharges in public sewerage systems, regulated
the maximum COD in 1600 mg/L and NH_4^+ in 60 mg/L, respectively, when specific
municipal regulations are not in force in the discharge point. Thus, this system, coupled
a polishing step featured in the aerobic MBR to provide high-quality characteristics to
the effluent.

In general, results indicated that the presence of the temperature control (stages II, III
and IV) impacted positively in the stability of the COD concentration in the UASB
effluent.

4.3.4.   Solids and nitrogen transformations in the MBR

Concerning TSS in the effluent, it was observed a full and sustained elimination due
to the porous size which retained solid particles, similarly to other researches operating
with MBRs (Buntner et al., 2013; Sánchez et al., 2016; Silva-Teira et al., 2017).
Although COD in the UASB effluent has been strongly changeable (Figure 4. 5), the
post-treatment showed a strong robustness since it was able to treat COD values from
a maximum of 3,052 mgCOD/L, when methanogenesis was inhibited at the operating
day 245, achieving a permeate concentration averaged in 55±65 mgCOD/L and
always fulfilling the discharge limits applied to the factory.

Similarly, solids production in the MBR has been strongly variable. The difference
between the COD in the UASB outlet and permeate acted as driver for VSS generation
in the MBR. Reported yields for VSS generation in MBR were between 0.2-0.4
kgVSS/kgCOD (Metcalf&Eddy et al., 2014; Silva-Teira et al., 2018) depending on the
applied Solids Retention Time (SRT). As permeate COD and observed overall
biomass yield could be estimated as constant values, biomass generation was
changeable and strongly dependent on the anaerobic UASB performance. Solids
management has been considered one of the major costs in the overall wastewater

treatment (Iglesias et al., 2017). The lower COD removed aerobically, the cheaper is its associated cost since the production rate has been at least 10 times lower in the anaerobic processes in comparison to an aerobic one. A quantitative estimation has been performed. Anaerobic and aerobic biomass yields are 0.03-0.18 (Lier et al., 2008) and 0.40-0.45 gVSS/gCOD$_{rem}$ (Metcalf&Eddy et al., 2014), respectively. Taking into account these yields and the experimental concentrations of COD removed via either anaerobic or aerobic processes, the overall estimation of solids production was 0.18 gVSS/gCOD$_{rem}$. In Stage I, sludge generation rate doubled the value obtained in the set II and III. Buntner et al., (2013) reported an overall yield of 0.07 gVSS/COD$_{rem}$ in a similar reactor and referred to the whole (UASB and MBR) system when operating with dairy wastewater. This reactor has been operated stably for 292 days in a temperature range of 17.5-24.5 ℃. A stable temperature has been important to maintain an optimal internal state of the UASB which in turn enhanced a high performance. The UASB behavior governed the operational treatment costs since the higher COD anaerobically removed: 1) the higher biogas (profitable as energy) gained and 2) the lower solids were generated (with a costly management associated).

Nitrogen transformations in the MBR have been tracked in (Figure 4. 8).
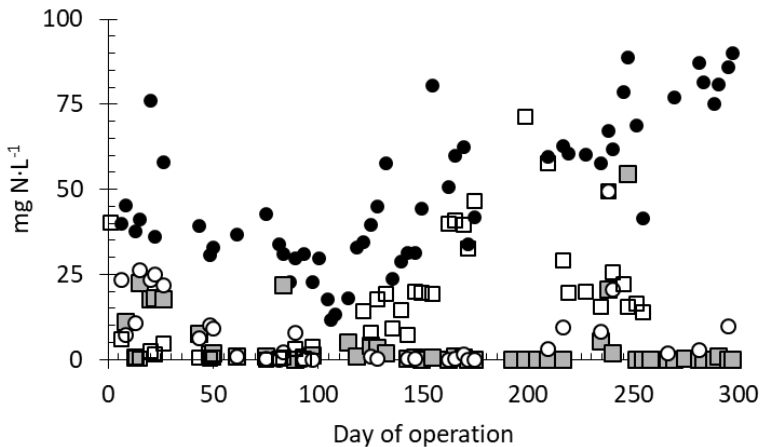
Figure 4. 8. Daily trends of nitrogen: as ammonium in the UASB outlet (●), as nitrate in the biofilm compartment (■), as nitrate in the permeate (○) and as ammonium in the permeate(□).

During the first 50 days of operation, a large extension of ammonium nitrification was observed. This fact was also viewed in a previous work employing the same system with dairy wastewater (Sánchez et al., 2013). From day 50 onwards, ammonium oxidizing capacity decreased. Two hypothesis were proposed for explaining this fact: 1) DO scarcity in both biofilm and MBR compartments and 2) QAC presence in the post-treatment. On the one hand, DO was scarce in the biofilm compartment due to a massive concentration of VSS which, in turn, required oxygen for endogenous activities. This fact led to DO concentrations of 0.0-1.0 mg/L in the biofilm compartment from day 50 to 200. Afterward, higher values were obtained, up to 1.5-6.0 mg/L. Nevertheless, this reason only may explain a lack of nitrification in the biofilm compartment since DO concentration in the MBR has been always higher than 3.0 mg/L due to the severe aeration demands of the membrane. For this reason, at least a fraction of nitrification was expectable in the MBR. The presence of QAC in the post-treatment could lead to a nitrification activity loss. This fact was according to (Sarkar et al., 2010) which studied the nitrification capacity losses in soils with concentrations of QAC as low as 50 mgQAC/kg soil. QAC presence inhibited the nitrification capacity at concentrations significantly lower than inhibitory thresholds for any other microbiological processes. Thus, this conclusion might explain the observed fact since QAC might not have been washed out to the non-inhibitory concentrations unlike the previous anaerobic process held in the UASB reactor. Nitrification capacity in the aerobic MBR was not recovered during the following 180 days of operation and it was only recovered in the last days of the experimental period, from day 200 onwards.

Nitrification batch assays were performed to elucidate the maximum nitrification capacity of the biomass taken from the MBR post-treatment. The obtained rates averaged a value as low as 3 mgN-NO$_3^-$/(L·d), confirming the lack of nitrification detected in the continuous operation. Sarkar et al., (2010) indicated the toxic effect of QAC to nitrifiers especially if the formed bonds are irreversible. The high DOC concentration in the mixed liquor could lead to a competition for the DO in which the heterotrophic biomass able to oxidize carbon competed in more favorable conditions than the autotrophic populations capable of oxidizing the ammonium present in the medium (Metcalf&Eddy et al., 2014).

4.3.5. Membrane performance

During the start-up and the first days of operation, the membrane behaved with a remarkable permeability, 356 ± 80 L/(m^2·h·bar), when the flux was in the range of 5-10 L/(m^2·h). Later, an increase of flux up to 14-17 L/(m^2·h) was setup. Once lower values were restored, the permeability decrease did not stop. From day 61 to 135, an overall permeability drop from the above-indicated values to those below 100 L/(m^2·h·bar) was observed. The fouling rate experimented in this period was 1.13 mbar/d.
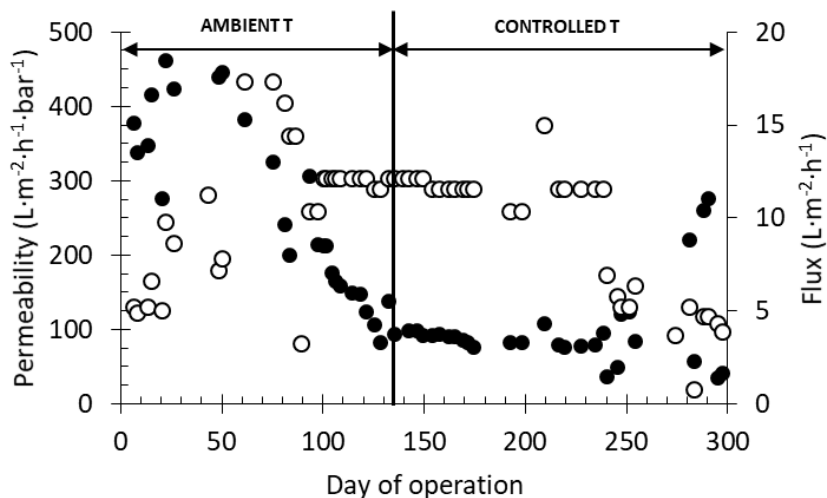
Figure 4. 9. Behavior of membrane parameters within the experimental period. In the main axis, permeability (corrected to 20ºC) was plotted (●). In the secondary axis, the evolution of the set flux (○) was depicted.

Apart from the excessive flux set by the permeate pump, observed fouling indicators monitored by the cBPC have been in the high range of 200 mgTOC/L (Sánchez et al., 2013). Thus, such a drop experimented in this period has been caused by a combined effect of two factors: 1) the above mentioned unexpected sudden jump in the flux, and 2) extremely high cBPC values (Figure 4. 10) up to 600 mgTOC/L, in comparison to a maximum of 100 mgTOC/L observed by Sánchez et al. (Sánchez et al., 2013).

From day 97 onwards, five intensive chemical cleaning attempts have been performed to try to recover the permeability. Permeability trends plotted in Figure 4. 9 indicated

that this set of trials have been unsuccessful and any permeability recover was not taken place. This incapacity of recovering the previous permeability by means of the intensive chemical cleanings indicated that this observed phenomena can be qualified as irrecoverable fouling (Drews, 2010).

Fouling parameters were additionally investigated. In Figure 4. 10 daily evolution of cBPC has been plotted.
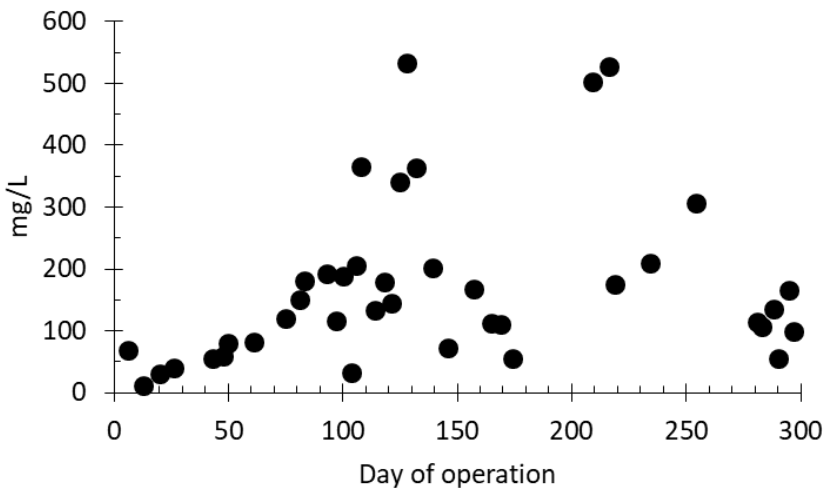

Figure 4. 10. Daily evolution of colloidal BPC (cBPC) within the experiments.

As the cBPC were assigned as the colloidal fraction of the dissolved organic carbon (DOC) of the sludge mixture in the liquid phase (Sánchez et al., 2013), the detected irrecoverable fouling can be caused by the cBPC high values, since the size of colloidal particles was similar than the pore size of the membranes and might block the pores, diminishing the filtration capacity.

From day 135 onwards, coincidently to the temperature control system enablement, permeability averaged to 88 ± 18 L/(m^2·h·bar). Unfortunately, these subdued values could not be increased due to: 1) the fouling has been qualified as irrecoverable (Drews, 2010), 2) cBPC were not diminished down to adequate values, maintaining a high fouling potential (Sánchez et al., 2013).

Cake resistance tests to the filtration capacity of the sludge were conducted with a calculated value of $1.10 \cdot 10^{12}$ m^{-1}. In this test, it has been determined that about the 35% of the overall resistance was a consequence of the colloidal fraction ($3.90 \cdot 10^{11}$ m^{-1}).

Other studies with a similar configuration than the employed in the current work and using the same model of membrane (Zenon ZW-10) improved the efficiency indicators. Silva-Teira et al., (2017) obtained a flux 12-15 L/(m^2·h) and a permeability 100-250 L/(m^2·h·bar). Buntner et al., (2013) observed a flux 13±3 L/(m^2·h) and a permeability 170±75 L/(m^2·h·bar).

In terms of microbiological indicators in the permeate, the presence of the ultrafiltration membrane has been considered one of the Best Available Technologies (BAT) for providing a high-quality permeate. An effluent free of microbial indicators was expected according to another study (Iglesias-Obelleiro et al., 2012) employing a similar pore size than the used in the current study. Even being out of the scope of this work, no microbial indicators were expected in the effluent and a full fulfillment of the Royal Decree 1620/2007 (Spanish Parliament) is expected. A further microbiological study will be required to confirm this information. Once doing so, treated water could be usable in the factory of origin for process and washing water, auxiliary water for cooling towers or evaporative condensers among other purposes.

4.4. Conclusions

The use of a combined two stages UASB and MBR system was used for treating an industrial wastewater stream generated in a seafood factory. The studied two-stages UASB + MBR was robust and reliable, and it has been capable of removing 94±4% of the incoming COD and the 100% of TSS present in the wastewater within the experimental period. The combined system was capable of counteracting the COD overloads to the MBR polishing system, when the UASB efficiency diminished. The COD values present in the permeate always fulfilled the discharge limits of the factory.

The presence of biocides in the industrial wastewater stream, in a concentration probably larger than 30 mg/L, inhibited both anaerobic treatment and nitrification

process. Methanogenic phase was recovered in the anaerobic treatment after 50 days and an irreversible nitrification drop in the post-treatment was experimented.

Temperature control and its stability over time have been essential parameters for achieving the maximum removed OLR (6 kgCOD/m^3·d) at 30 ºC, corresponding to more than the 90% of the fed OLR in the UASB system. Observed methanation percentages were larger than 90%. In these conditions, larger removal performances could be reached, since the OLR fed to the UASB was limited by the incoming wastewater COD concentration and the filtration capacity of the membrane.

Chapter 5

Removal of dissolved methane and nitrogen from anaerobically treated effluents at low temperature by MBR post-treatment[2]

Summary

Sewage treated anaerobically at low temperature contains dissolved methane, which should be removed in order to reduce greenhouse gas emissions (GHG). In this research, a Membrane Bioreactor (MBR) post-treatment was proposed that is able to simultaneously remove methane and nitrogen by implementing newly discovered biological processes involving methane oxidation coupled to denitrification. Up to 95% of methane was removed at 17-23 °C. Moreover, biological treatment partially removed nitrogen, up to 15-20 mg TN/L, by coupling methane oxidation and denitrification. This study opens the door to reducing the GHG impacts associated to the anaerobic treatment of sewage in temperate and warm climates countries. The elimination of the majority of the dissolved methane, suspended solids and the remaining biodegradable COD of the anaerobically treated effluents, converted this treatment as friendly from the ecological point of view, reducing part of the nitrogen contained in sewage.

5.1. Introduction

Anaerobic wastewater treatment has been considered a suitable option for treating municipal wastewater in areas with warm climates. COD removal rates ranging from 60 to 80% can be achieved when applying anaerobic wastewater treatment at 20-25 °C and with organic loading rates (OLR) of approximately 2-3 kg COD/($m^3 \cdot d$) (Lier et al., 2015; Ozgun et al., 2013). In the last few years, research on anaerobic treatment of wastewater has been considered a promising research area as a consequence of its attractive benefits. The absence of aeration, lower sludge production and its applicability over a wider range of OLR values compared to traditional technologies such as conventional activated sludge (CAS) make this treatment worthy of further investigation. Moreover, methane-rich biogas is obtained that can be profitably used to produce energy.

In anaerobic conditions, a considerable fraction of the total methane produced, more than 60% at low temperatures, is dissolved in the effluent (Noyola et al., 2006). In the case of sewage treatment, a range between 20 to 60% has been reported (Souza et al., 2011). Methane can be easily stripped off in the typical aerobic post-treatment, as observed in a study of GHG emissions after anaerobic treatment in a full-scale WWTP in Japan (Masuda et al., 2014). Methane has been classified as a harmful GHG, with a warming potential 34 times that of CO_2 in a 100-year scenario. Methane related to anaerobic waste and wastewater treatment is responsible for 2.8% of the world' overall GHG emissions (Myhre et al., 2013). This fact reflects a considerable environmental issue, one that argues against the use of anaerobic technologies compared with CAS treatments (Cakir and Stenstrom, 2005). To diminish these GHG emissions, the dissolved methane present in anaerobically treated effluents should be eliminated, thereby addressing the targets set forth during the 2015 Climate Change Conference in Paris (United Nations, 2015).

To reduce the impacts of anaerobic treatments, alternative post-treatments should be installed. There are expensive alternatives already available. One option is the combustion of stripped-off methane; another is catalytic methane oxidation, well known for its remarkable efficiency (Noyola et al., 2006). Recently, biological solutions
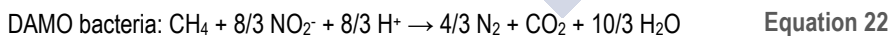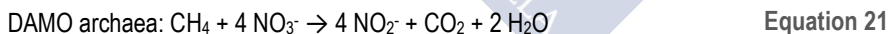
are being developed to reduce methane content that act directly in the effluent (Hatamoto et al., 2011; Matsuura et al., 2015).

At the end of the last century, it was demonstrated the use of methane as a carbon source for biological denitrification (Thalasso et al., 1997). The overall process can be accomplished by using aerobic or anaerobic methane-oxidizing microorganisms (Modin et al., 2007). Aerobic methanotrophs are able to convert methane into oxidized species such as methanol, formaldehyde or acetate, compounds that are fully soluble in water. These methane oxidation products can be employed as a carbon source by heterotrophic denitrifying microorganisms in a subsequent reaction. This process is known as aerobic methane oxidation coupled to denitrification (AMO-D) and has been described by the summarized reaction below (Zhu et al., 2016).

$$CH_4 + 1.1\ O_2 + 0.72\ NO_3^- + 0.72\ H^+ \rightarrow 0.36\ N_2 + CO_2 + 2.36\ H_2O \qquad \textbf{Equation 20}$$

Modin et al. (Modin et al., 2008) developed a lab scale membrane biofilm reactor (MBfR) with a liquid volume of 0.8 L, achieving elimination rates of 21 and 8 mg/(L·d) of methane and nitrogen, respectively, exclusively through this via.

In the absence of oxygen, specific, recently discovered microorganisms are able to couple denitrification to anaerobic methane oxidation (DAMO) (Modin et al., 2007). This process can be carried out either using nitrite and DAMO bacteria (*Candidatus Methylomirabilis oxyfera*) or nitrate and DAMO archaea (*C. Methanoperedens nitroreducens*) (Haroon et al., 2013).

$$\text{DAMO archaea: } CH_4 + 4\ NO_3^- \rightarrow 4\ NO_2^- + CO_2 + 2\ H_2O \qquad \textbf{Equation 21}$$

$$\text{DAMO bacteria: } CH_4 + 8/3\ NO_2^- + 8/3\ H^+ \rightarrow 4/3\ N_2 + CO_2 + 10/3\ H_2O \qquad \textbf{Equation 22}$$

A consortium of anammox, DAMO archaea and DAMO bacteria was discovered (Haroon et al., 2013). DAMO archaea were responsible for reducing nitrate into nitrite, and then anammox and DAMO bacteria competed for nitrite. In the long term, it seems that the anammox bacteria outcompete the DAMO bacteria and the latter tend to disappear (Hu et al., 2015). The simplified stoichiometry of the anammox process is summarized below (Jetten et al., 1997).
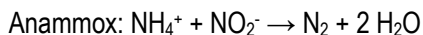
Anammox: $NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O$ **Equation 23**

Unfortunately, these newly discovered DAMO microorganisms are characterized by extremely slow growth rates of approximately 1-2 weeks (Ettwig et al., 2009). Moreover, it seems that the capacity of DAMO enrichment reactors, which relies on sedimentation of the biomass from the treated water, could be limited by the tendency of these microorganisms to be washed out with the effluent. Kampman et al., (2012) conducted an enrichment experiment in a sequencing batch reactor and stated that DAMO microorganisms were washed-out with the effluent, limiting the nitrogen removal rate below 35 mgN/(L·d) enrichment. Due to this fact, bioreactor configurations for preventing biomass washout, such as membrane filtration or biofilms, have to be considered. This problem has been overcome by installing a membrane, resulting in a maximum nitrogen removal rate (NRR) of 36 mgN/(L·d) (Kampman et al., 2014). Very recently, it was observed at lab scale a DAMO activity of 40 mgN/(L·d) (Bhattacharjee et al., 2016). Moreover, it was developed a lab-scale hollow fiber membrane biofilm reactor with a surprisingly high NRR of 684 mgN/(L·d) (Cai et al., 2015). This value has not been replicated in further studies at similar or larger scales. In a previous study, it was studied the same system than in the current work, using dissolved methane as an electron donor for denitrification in a 180 L pilot-scale UASB with a pre-anoxic MBR system (Sánchez et al., 2016). Methane removal rates (MRR) were approximately 150 mgCH$_4$/(L·d). NRR of 79 mgN/(L·d) were observed, although different nitrogen removal processes were observed.

With this outline, the aim of this study was to demonstrate the feasibility of an innovative bench-scale MBR post-treatment for the "eco-friendly" treatment of low-strength UASB effluents at approximately 20 °C. This work focused intensively on minimizing the GHG emissions associated with dissolved methane which appears in the effluent of UASB reactors at these relatively low temperatures. To reach this goal, a pre-anoxic MBR post-treatment has been developed to treat synthetic, anaerobically treated municipal sewage that removes methane and nitrogen simultaneously.

5.2. Materials and methods

5.2.1. Experimental setup

A schematic of the bench-scale system is shown in Figure 5. 1. The methanogenic treatment was conducted in a UASB reactor of 120 L. The pre-anoxic MBR (56 L) was composed of two compartments. The first compartment (36 L) was mechanically stirred, and is denoted as the pre-anoxic compartment hereafter. In this compartment, biomass was present both in suspension and adhered onto foam carriers (LEVAPOR biocarrier made by LEVAPOR GmbH Biofilm Technologies, Leverkusen-Germany). 20% of the apparent volume was occupied by the supports. These carriers were selected due to their ability to promote an anaerobic environment in the inner parts of the foamy material.
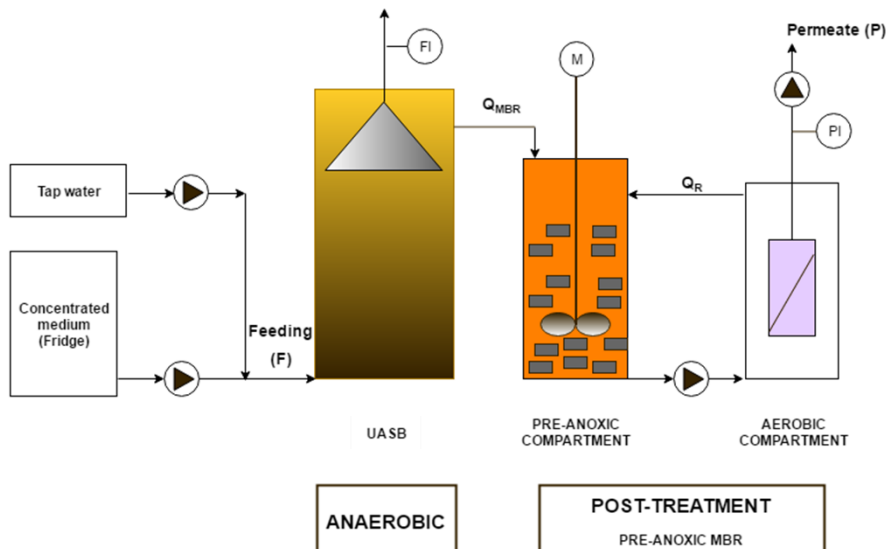
Figure 5. 1. Schematic diagram of the pilot plant composed by an anaerobic stage (UASB methanogenic pre-treatment) and a MBR post-treatment consisting of a pre-anoxic compartment and an aerobic compartment where the membrane was located.

The second, aerated compartment (20 L), known as the aerobic compartment hereafter, contained an ultrafiltration hollow fiber membrane (Zenon ZW-10, made by

GE Water Treatment Systems & Process Technologies) with an effective filtration surface of 0.9 m². Physical separation between the mixed liquor and permeate was achieved using a membrane with a pore diameter of 0.04 μm. The permeation cycle was 7.5 min long, and included 0.5 min of backwashing and 7 min of filtration. This compartment was aerated with a specific air demand (SAD_m) of 0.7 m³/(m²·h) to minimize membrane fouling. The transferred oxygen was employed to oxidize the ammonium to nitrite and nitrate. The operation of the system was monitored by a PLC (Siemens S7-200) connected to a computer. Transmembrane pressure (TMP) data was measured with an analog pressure sensor (IFM Efector500 PN-2009) and collected in the PC by an analog PLC module (Siemens EM 235).

An internal recycle stream (R) connecting the aerobic to the pre-anoxic compartment was set up to allow communication between the compartments. The system was punctually purged when the Total Suspended Solids (TSS) content was relatively high. The mass of TSS wasted was measured and taken into account to estimate the overall biomass yield (Y_{OBS}).

The system was fed with a synthetic low-strength wastewater. A concentrated medium made up of skim milk, $NaHCO_3$ (200 mg/L), NH_4Cl (9.3 mgN/L) and trace amounts of $FeCl_3·6H_2O$ (1.5 mg/L), H_3BO_3 (0.15 mg/L), $CuSO_4·5H_2O$ (0.03 mg/L), KI (0.03 mg/L), $ZnSO_4·7H_2O$ (0.12 mg/L), $CoCl_2·6H_2O$ (0.15 mg/L), $MnCl_2·4H_2O$ (0.12 mg/L) was prepared.

This experimentation has been carried out immediately after the previous research in the same system (Sánchez et al., 2016), that was a first attempt to tackle the removal of methane and nitrogen from UASB effluents, using an MBR post-treatment.

5.2.2. Analytical methods

Volatile Suspended Solids (VSS), total and soluble chemical oxygen demand (COD), nitrite, nitrate and ammonium were determined according to the Standard Methods (Rice et al., 2012). Total nitrogen (TN) concentration was measured with a kit (Spectroquant Cell Test made by Merck). Temperature and dissolved oxygen (DO) were measured with a multi-parameter meter (Hach HQ40d) with a luminescent optical probe (IntelliCAL LDO101). Oxidation reduction potential (ORP) was continually

monitored with a probe (Hach DRD1P5) connected to a controller (SC100) of the same brand. A portable pH meter (Crison PH-25) was employed. Biogas production was measured by using a Milli GasCounter MGC-10 (Ritter, Germany) and its composition was measured in a gas chromatograph (HP 5890 Series II) with the column of Porapack Q 80/100 2 m x 1/8" (SUPELCO).

Dissolved methane in the liquid phase was estimated by an adaptation of the method previously proposed (Souza et al., 2011). The protocol was carried out as follows. (1) A sample of approximately 300 mL was taken from both the UASB and pre-anoxic compartment outlets. The volume of the sample was measured precisely, and the samples were then weighed. (2) Samples were hand-shaken in watertight 500 mL Pyrex flasks. (3) After three minutes of shaking, the exsolved methane to gas phase was analyzed in the gas chromatograph. (4) The amount of dissolved methane was estimated using equilibrium calculations for gas/liquid phases, by means of Henry´s law, Equation 24.

$$x_{CH_4} = k_H(T) \cdot p_{CH_4} \left[mol/_{kg} \right]$$

<div align="right">**Equation 24**</div>

where x_{CH4} is the concentration of methane in liquid phase; $k_H(T)$ is the Henry´s constant and p_{CH4} is the partial pressure of methane in the gas phase.

Henry's equilibrium parameters were obtained from the website of the US National Institute of Standards and Technology (NIST) (http://webbook.nist.gov/cgi/cbook.cgi?ID=C74828&Mask=10). Equation 24 and Equation 25 were employed to determine the amount of dissolved methane in the liquid phase as a function of the temperature and the partial pressure of methane in the gas phase.

$$k_H(T) = k_H^0 \cdot exp \cdot \left\{ A \cdot \left[\left(\frac{1}{T} \right) - \frac{1}{298.15} \right] \right\} \left[\frac{mol}{kg} \cdot bar \right]$$

<div align="right">**Equation 25**</div>

where tabulated values of A = 1600 and k_H^0 (Henry's law constant at 298.15 K) = 0.0014 mol·bar/kg were used.

5.2.3. Operational strategy

The internal recycle ratio (R) of the post-treatment is described by Equation 26. Q_R and F streams are graphically identified in Figure 5. 1.

$$R = \frac{Q_R}{F}$$

<div align="right">Equation 26</div>

where Q_R is the flow back from the aerobic to the pre-anoxic compartments and F is the synthetic medium stream fed to the system.

R was varied in steps during nine different periods by setting the flow of the internal pump which moved material from the pre-anoxic to the aerobic compartments of the post-treatment MBR (Figure 5. 1). This procedure was carried out to evaluate the impact of this parameter on MBR removal performance in terms of methane and nitrogen removal. Table 5. 1 contains the operation days and the R-value set during each period. The inlet flow was maintained constant in all experimental periods except the one corresponding to R of 2.8, where it was increased by 40% to assess the impact of both methane and nitrogen loads in the system.

Table 5. 1. Description of the days of operation and number of samples included in each of the experimental periods

R	Days of operation	Samples	Inlet flow (L/h)
0.5	0-35	15	10.6
0.8	36-70	18	10.6
1.2	71-106	6	10.6
1.8	107-142	5	10.6
2	143-178	6	10.6
2.8	287-323	9	14.8
3.1	215-250	12	10.6
3.4	251-286	7	10.6
4.0	179-214	8	10.6

Concerning the input of DO to the pre-anoxic compartment (Figure 5. 1), the oxygen inputs to the pre-anoxic compartment were the recycle stream R coming from the aerobic compartment and the oxygen transferred from the environment through the top part of the pre-anoxic compartment. This second factor was governed by the oxygen transfer coefficient (k_La^{O2}) of the system, and it did not vary over time since the stirring speed was held constant. The oxygen removal rate (ORR) is described in the Calculations section, below, and it was used to estimate mass balances, methane and nitrogen removal through AMO-D.

The methane transferred to the environment was governed by the methane transfer coefficient (k_La^{CH4}). Moreover, the oxygen transfer coefficient (k_La^{O2}) was estimated considering the value of the methane transfer coefficient, which was experimentally determined, as previously described (Sánchez et al., 2016).

5.2.4. Fluorescence in situ hybridization (FISH)

Biomass (2-4 mL) was harvested from the pre-anoxic compartment on the 215th day of operation to determine which microorganisms were present. The culture was washed with phosphate-buffered (PBS 10 mM) saline and fixed for 3 h with paraformaldehyde. After an extra wash, the samples were stored at -20 °C. For microscopy, fixed samples were dehydrated in 50, 80, and 96% ethanol for 3 minutes each and hybridized. The probes employed for detecting the corresponding microorganisms were as follows: type I aerobic methanotrophs (MG84 and MG705); type II aerobic methanotrophs (MA450); DAMO bacteria belonging to the NC10 phylum (DBACTmix: DBACT193 + DBACT1027); DAMO archaea (DARCH872); anammox: (amx368 + amx820).

5.2.5. Batch assays

Batch assays were carried out by adapting the methodology proposed in a previous research (Sánchez et al., 2016). To evaluate the influence of COD and CH_4 concentrations as electron donors in the DAMO anaerobic processes, 8 different 500 mL Pyrex flasks were filled with 300 mL biomass and biofilm carriers in the same proportion as that in the pre-anoxic compartment. Both suspended biomass and biofilm carriers were taken directly from the pre-anoxic MBR. To guarantee the absence of

biodegradable organic matter, suspended biomass and biofilm carriers were rinsed three times with phosphate buffer solution.

Every bottle assay contained 1.16 gVS of biomass in 300 mL of liquid phase, including suspended biomass and biomass attached to the biofilm carriers. Nitrate was added to obtain an initial concentration of 30 mgN-NO$_3^-$/L in the flasks, emulating conditions in the pre-anoxic compartment. To eliminate the oxygen initially present in the gas phase, a stream of either nitrogen or methane gas was flushed into the headspace until the total displacement of the oxygen was guaranteed. The pH was adjusted to a value of 7.0 by adding either HCl or NaOH. Flasks were sealed and shaken at 150 rpm during 8 h at 20 °C. The liquid phase was sampled every hour to analyze the evolution of nitrogen species.

The batch assays were designed for quantifying different denitrification mechanisms expected in the studied system. In case study I (endogenous activity), neither methane nor external COD was added, and this case study consisted of one of the bottles. In case study II (heterotrophic activity), external COD was added to each flask as sodium acetate varied from 20 to 90 mgCOD/L, relative to the liquid phase. In case study III (endogenous + heterotrophic + methane denitrification), the liquid medium from 4 out of the 8 bottles was bubbled with CH$_4$ to saturation.

In case study I (endogenous activity), only heterotrophic denitrification occurred due to endogenous activity that employed decay products as its electron source.

In case study II (endogenous + heterotrophic activities), denitrification was due to two factors: endogenous activity and the presence of organic matter that acted as a reduction agent. In both cases, denitrification took place via heterotrophic denitrifiers.

In case study III (endogenous + heterotrophic + methane denitrification), nitrogen removal occurred by means of endogenous activity, heterotrophic denitrification and methane related mechanisms (He et al., 2015; Sánchez et al., 2016). These experiments are summarized in Table 5. 2. In this case, the denitrifying activity was due to the sum of both heterotrophic denitrifiers and DAMO microorganisms.

Table 5. 2. Summary of the batch assays performed to determine the DAMO activity.

	Case study		
	I	II	III
Denitrification via	Endogenous	Endogenous + heterotrophic	Endogenous + heterotrophic + methane
Number of bottles	1	3	4
Acetate (mgCOD/L)	-	20-50-90	0-20-50-90
CH₄	-	-	Liquid phase saturation

Additionally to the above described batch assays, anammox activity tests were performed as previously described (Dapena-Mora et al., 2007).

5.2.6.   Calculations

Removal rates of oxygen, methane and nitrogen oxidized anions were calculated by applying mass balances to the pre-anoxic compartment. The balances were referred to this compartment in which nitrogen anions and methane removal took place. It was taken into consideration that the approximation to an ideal CSTR can be applied to this compartment. NRR was calculated according to Equation 27:

$$NRR = \frac{F \cdot R \cdot \{C_{NO3}^{aer} - [C_{NO3}^{anox} \cdot (1 + 1/R)]\}}{V_R} \qquad \text{Equation 27}$$

where the superscript indexes *aer* and *anox* denote the aerobic and pre-anoxic compartments, respectively; V_R is the volume of the pre-anoxic compartment; and F is the feeding flow. Likewise, MRR was calculated by means of Equation 28:

$$MRR = \frac{F \cdot \{C_{CH4}^{UASB} - [(1 + 1/R) \cdot C_{CH4}^{anox}]\} - k_L a_{CH4} \cdot C_{CH4}^{anox}}{V_R} \qquad \text{Equation 28}$$

where C_{CH4}^{i} indicates the methane concentration either in the UASB outlet or in the pre-anoxic compartment, as previously indicated.

Additionally, methane removal percentage (%CH₄) was calculated as indicated in Equation 29:

$$\%CH_4 = 1 - \left[(R + 1) \cdot \frac{C_{CH4}^{anox}}{C_{CH4}^{MBR}}\right]$$

Equation 29

Similarly to NRR and MRR, ORR was determined by means of Equation 30:

$$ORR = \frac{F \cdot R \cdot \{C_{O2}^{aer} - [C_{O2}^{anox} \cdot (1 + 1/R)]\} + k_L a_{O2} \cdot (C_{O2}^{*} - C_{O2}^{anox})}{V_R}$$

Equation 30

where C_{O2}^{i} represents the DO concentration in either the aerobic (aer) or pre-anoxic compartments (anox) or its concentration of saturation in water (*).

The statistical analyses were performed using SPSS Statistics software, version 21.0.

5.3. Results and discussion

5.3.1. General results

The concentrations of COD_T, COD_S, and TN in the wastewater feed were 552±153, 471±150 and 34±9 mg/L, respectively. During the experiments, the UASB system was operated at a temperature of 21±2 °C and a pH of 6.7±0.3. HRT was maintained between 10 and 12 h in the UASB, and 4.6 and 5.6 h in the MBR post-treatment.

The methanogenic treatment led to a stable COD removal of 84±9% in the anaerobic stage. The measured biogas production was 37±8 L/d with a methane percentage of 79±2%. The concentration of dissolved methane measured in the UASB-treated wastewater was 29±4 mgCH₄/L, and it was approximately constant during the continuous operation. COD balances in the UASB, elucidated that around 90% of the removed COD was methanized. Methane mass balances indicate that dissolved methane corresponded to 20-30% of the overall methane generated in the UASB reactor.

The effluent from the UASB was post-treated in the pre-anoxic MBR system. The concentrations of the main constituents of the UASB effluent were as follows: COD_T 86±47 and COD_S 35±19 mgCOD/L, excluding dissolved methane in both cases. The

TN concentration was 36±10 mgN/L, which is similar to the measured ammonium concentration of 34±9 mgN-NH$_4^+$/L.

TSS and VSS concentrations were 6.4±1.5 and 5.7±4.2 g/L, respectively, in the pre-anoxic compartment. DO and ORP were constantly monitored. These parameters had values that ranged between 0.1 and 0.2 mgO$_2$/L, were < 0 mV respectively, during the whole experimental period. The pH was around 6.7±0.3. TSS and VSS concentrations of 8.6±2.0 and 7.8±1.7 g/L were observed in the aerobic MBR compartment, and DO was 2.8±1.5 mgO$_2$/L. When measuring DO concentrations lower than 0.1 mg/L, available DO probes might not be reliable. In these conditions, ORP may be an accurate tool for describing anoxic environments (Lie and Welander, 1994). ORP in the pre-anoxic compartment varied between -50 and -200 mV indicating DO depletion in the pre-anoxic compartment (Saby et al., 2003).

The observed overall biomass yield (Y$_{OBS}$) in the whole UASB and MBR was 0.10-0.14 gVSS/gCOD. The remaining COD present in the permeate was approximately constant, 12±14 mgCOD/L, and the nitrogen ion concentration strongly depended on the experimental conditions. Suspended solids were totally removed from the wastewater due to the membrane's retention capacity.

Regarding the membrane performance, net flux (always corrected with the backflush) was maintained at 12-15 L/(m^2·h), TMP varied around 69±18 mbar and permeability values ranged between 100-250 L/(m^2·h·bar). These values were similar to those obtained in previous studies that employed a ZW10 membrane in similar configurations (Buntner et al., 2013; Sánchez et al., 2016).

5.3.2. Impact of R on dissolved methane removal

The internal recycle ratio (R) of the post-treatment was one of the most important operating parameters affecting the reactor's performance. R affected the amount of nitrogen anions and DO recycled from the aerobic to the pre-anoxic compartment, among other factors. The loading rates of organic material (excluding methane), methane and nitrogen fed to the MBR post-treatment were held approximately constant, except in the period corresponding to R = 2.8 were the inlet flow was 40% incremented to assess the impact of the methane load in the system. R was raised in

steps from 0.5 to 4.0 to assess its impact on methane removal, as indicated in Table 5. 1. The evolution of dissolved methane concentration fed to the MBR post-treatment, and in the pre-anoxic compartment has been depicted in Figure 5. 2.
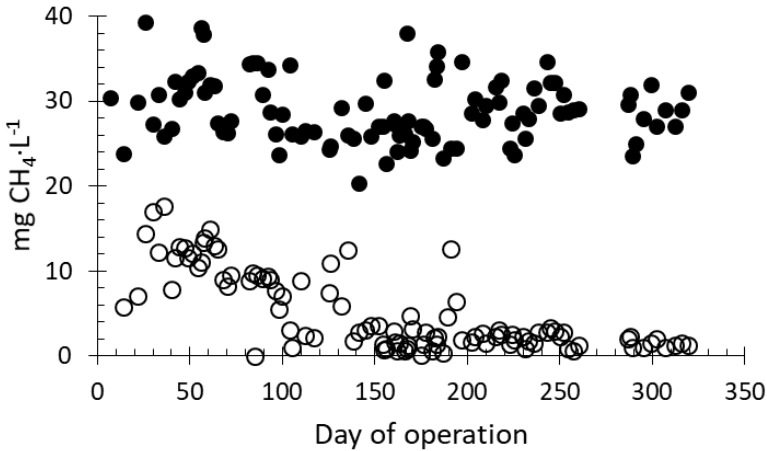

Figure 5. 2. Evolution of the dissolved methane concentration fed to the MBR post-treatment (●) and measured in the pre-anoxic compartment (○)
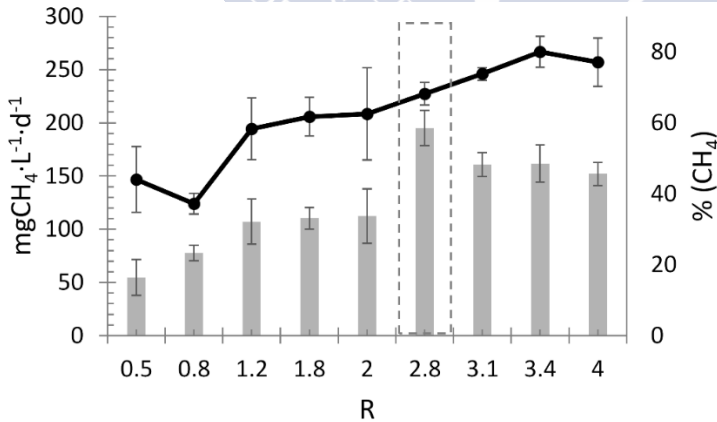

Figure 5. 3. Effect of recycle ratio (R) on the calculated methane removal rate MRR (●), in main axis, and methane elimination percentages (■), in secondary axis. The framed area denotes the period when the inlet flow was increased in a 40% when R was set at 2.8.

The collected data in Figure 5. 2 as long as the determined k_La^{CH4} value of 0.78 d^{-1} were employed to reckon MRR and methane removal percentage values. Figure 5. 3 summarizes the observed results of % CH_4 and MRR relative to the pre-anoxic compartment volume. Methane removal percentage increased gradually with R, from 37 to 80%, when the inlet flow was fixed at 10.6 $L \cdot h^{-1}$. The highest average removal percentages were observed at R values set from 3.1 onwards. Thus, in these conditions, the maximum removal percentage was limited to 80%.

Nevertheless, the methane removal rate followed a slightly different behavior. The experimental results showed three different elimination levels as a function of this parameter. The lowest elimination values were observed when R varied between 0.5 and 2.0. MRR were never larger than 112±36 mgCH$_4$/(L·d), corresponding to a methane removal percentage of 62%. A maximum rate of 195±17 mgCH$_4$/(L·d) (removal percentage of 68%) was detected when R was set to 2.8, corresponding to the final stage of the experimental period. During this period, the feed flow rate was increased approximately 40%, to evaluate the impact of the dissolved methane load on the efficiency. For R values of 3.1 and greater (again with the flow set at 10.6 L/h) a slight decrease was observed until MRR reached 152±17 mgCH$_4$/(L·d) with a methane removal percentage of 75-80%. Considering this information, the results showed that the optimal R-value was between approximately 2.8 and 3.4, in terms of methane elimination. Moreover, the observed behavior in presence of a higher flow of 14.8 L/h (R of 2.8) elucidated that the system would have been able to operate at higher capacity than the set flow of 10.6 L/h since the MRR was increased when the methane load was augmented and % CH_4 was not significantly affected.

Table 5. 3 depicts a comparison between the above discussed results and a study held on the same pilot feeding a higher strength wastewater. Sánchez et al. (2016) only included data for low R-values, from 0.5 to 2.0.

Table 5. 3. Methane removal ratio comparison between Sánchez et al. (2016) and the current study.

R	MRR mgCH$_4$/(L·d)	
	Sánchez et al. (2013)	Current study (2017)
0.5-1.0	80 ± 10	20 ± 10
1.5-2.0	40 ± 15	30 ± 15

A different behavior was observed in Sánchez et al. (2016) if compared with the current one. The lower R the higher MRR was stated by Sánchez et al (2016). A methane elimination percentage over 80% was observed when R was set at 0.5-1.0. In the current research, when equal R-values were set, methane percentage values as low as 20-30% were attained.

The main difference between both experiments was the COD_T fed into the pre-anoxic MBR. UASB effluent concentrations were 200-300 mgCOD/L in Sánchez et al. (2016) and 86-120 mgCOD/L in the current study. A higher COD concentration observed in the UASB effluent promoted a larger extension of the denitrification through the conventional heterotrophic via.

Table 5. 4 shows a compilation of previously published information on MRR, as well as the microbiological pathways involved. These data indicated that the proposed system, which operates at bench scale, showed slightly better results than those reported in previous studies. In terms of MRR, the maximum observed value of 195±17 mgCH$_4$/(L·d) in this work is the highest of the available values for treating dissolved methane in liquid phases. Significantly lower values of 125 mgCH$_4$/(L·d) were reported when synthetic wastewater was fed into a down-flow hanging sponge (DHS) reactor under aerobic conditions to simulate inputs of anaerobically treated effluents containing dissolved methane in the liquid phase (Hatamoto et al., 2011). In a previous study performed in our laboratory but using AnoxKaldnes® carrier and a significantly higher-strength wastewater of 261 mgCOD/L, compared with the 85.9 mgCOD/L wastewater used in the present study. It was observed 153 mgCH$_4$/(L·d) in the same experimental system (Sánchez et al., 2016). A newly discovered microorganism, included in the NC10 phylum but distinct from *Candidatus Methylomirabilis oxyfera*, was harvested in a lab-scale study with biomass inoculated from a natural ecosystem, and was able to eliminate 3-4 mgCH$_4$/(L·d) using pure cultures of the microorganisms (Bhattacharjee et al., 2016). This activity value is still much lower than that observed in the current study.

Table 5. 4. Summary of the available data in the literature for methane and nitrogen removal rates for AMO-D and DAMO pathways

	MRR (mgCH$_4$·L^{-1}·d^{-1})	%CH$_4$	NRR (mgN·L^{-1}·d^{-1})	Heterotrophic denitrification	Microbiological pathway	System and volume	Observations
Thalasso et al. (1997)			600 (mgN·kgVSS^{-1}·d^{-1})		AMO	Batch assays, 0.4 L	CH$_4$ removal percentage not determined
Hatamoto et al. (2011)	125	95	0		AMO	Down-flow hanging sponge (DHS), 4 L	Methane aerobically mineralized No nitrogen removal
Shi et al. (2013)	Not shown		190		DAMO + anammox	Membrane biofilm reactor, 1.15 L	Growth medium for DAMO
Cai et al. (2015)	Not shown		684		DAMO + anammox	Membrane biofilm reactor, 1.15 L	Growth medium for DAMO
Matsuura et al. (2015)	Not shown	99	Ammonium oxidation			Double down-flow hanging sponge (DHS), 155 L	Methane aerobically mineralized No nitrogen removal
Sánchez et al. (2016)	153	95	79	Probably high	AMO-D + DAMO + anammox	Pre-anoxic MBR AnoxKaldnes carriers, 56 L	Soluble COD fed 57±35 mg/L
Bhattacharjee et al. (2016)	3		40		DAMO	Discontinuous sequencing batch reactor (SBR), 1.9 L	Growth medium for DAMO
Current study	180	80	150	Partially present	AMO-D + DAMO	Pre-anoxic MBR Levapor carriers; low strength wastewater, 56 L	Soluble COD fed 35±19 mg/L

5.3.3. Nitrogen removal in the system

As previously stated, the role of the R value has been considered crucial. This parameter governs not only parameters such as the hydraulic contact time in the pre-anoxic compartment but also the amount of oxygen or nitrogen anions recycled from the aerobic to the pre-anoxic compartments. Thus, methane/-nitrogen interactions undoubtedly varied when R was modified.

UASB effluent with an ammonium concentration of 34 ± 9 mgN-NH_4^+/L was directed into the MBR post-treatment. Nitrogen ions concentration in the permeate were 0.8 ± 0.2 mgN-NH_4^+/L, 0.1 ± 0.1 mgN-NO_2^-/L and 25 ± 7 mgN-NO_3^-/L. In Figure 5. 4, both nitrogen inflow and outflow trends in the MBR post-treatment were represented. In the aerobic compartment, measured DO concentrations ranged from 1.0 to 5.1 mg/L. The average DO concentration and its standard deviation were 2.8 ± 1.5 mgO_2/L. The measured DO in the pre-anoxic compartment was negligible (0.1 ± 0.1 mgO_2/L). Moreover, redox potentials indicated a strictly anoxic environment in this compartment. This result indicated that ammonium was almost fully oxidized to nitrate in the aerobic compartment.
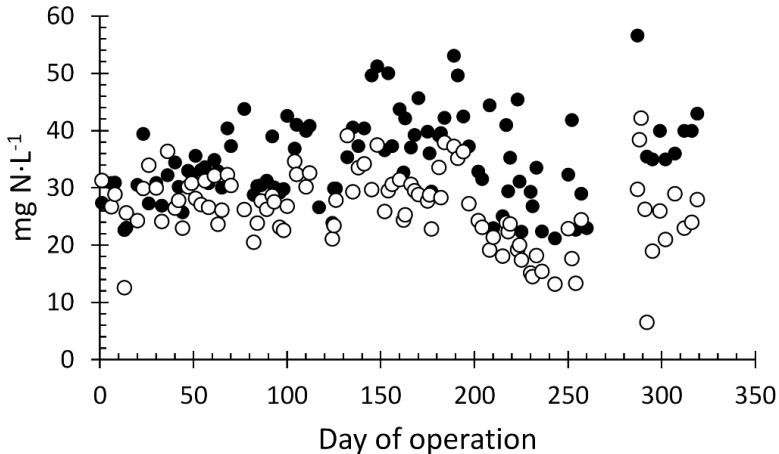
Figure 5. 4. Evolution of ammonium concentration fed to the MBR post-treatment (●) and total nitrogen ions concentration the permeate (○).
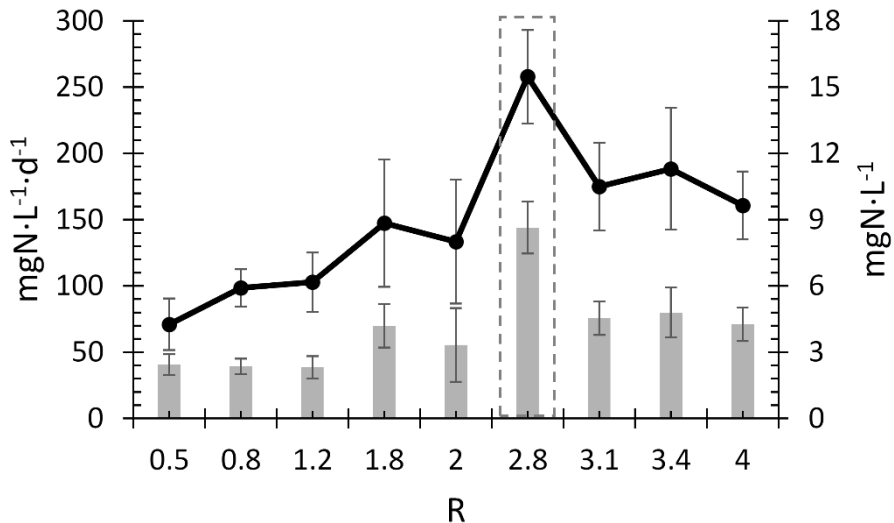
Figure 5. 5. Effect of recycle ratio (R) on nitrogen removal rate NRR (▪), in main axis, and eliminated (●), nitrogen concentration, in secondary axis. The framed area denotes the period when the inlet flow was increased in a 40% corresponding to R = 2.8.

The nitrogen anions detected in the aerobic compartment were recirculated to the pre-anoxic compartment through the recycle stream to complete the nitrogen denitrification cycle. Average nitrogen ions concentrations measured in both the pre-anoxic compartment (27±8 mgN/L) and the permeate (26±7 mgN/L) were similar, and they were lower than the total nitrogen in the effluent of the UASB system (34±10 mgN/L), confirming that nitrogen denitrification was restricted to the pre-anoxic compartment.

As with methane, nitrogen elimination was studied as a function of R using the same values and nitrogen load (by governing the inlet flow). In Figure 5. 5, the main results are summarized. NRR showed large variability between 39-144 mg N/(L·d), relative to the pre-anoxic compartment volume. The lowest NRR values were observed at the lowest R values, with NRR just over 40 mgN/(L·d) for values of R between 0.5 and 1.2. In contrast, for R values of 3.1 or greater, nitrogen removal stabilized at 70-80 mgN/(L·d). An unusually high NRR value of 144±20 mgN/(L·d) was observed when R was set at 2.8 (period in which the flow increased 40%). This rate was approximately 100% higher than those observed with the other R values between 1.8 and 4. This result was in accordance to the incremented MRR observed in Figure 5. 3 in the same

period, since both nitrogen and methane eliminations have been related by Equation 20, Equation 21 and Equation 22. Nitrogen concentration removed increased gradually with R, following a similar pattern as NRR. The best performance was also observed at an R value of 2.8. This could be a result of the higher methane loading rate fed to the pre-anoxic compartment, which raised the observed denitrification rate.

With regards to nitrogen denitrification, not only was methane present as an electron donor (AMO-D and DAMO), but also the remaining COD coming from the UASB reactor (heterotrophic denitrification) and ammonium (anammox). Considering the possibility of heterotrophic nitrogen denitrification, volatile fatty acids (VFA) and biodegradable COD coming from the UASB reactor might act as carbon sources. However, VFA is negligible because the measured values were below the detection limit of 20 mg/L. The difference between the soluble COD present at the pre-anoxic MBR inlet and in the effluent (35±19 and 12±14 total and soluble, respectively, expressed in mgCOD/L) could account for a possible nitrogen elimination of 4 mgTN/L, considering a 5 gCOD/gN for denitrification (Metcalf&Eddy et al., 2014).

The overall nitrogen removed was up to 15 mgTN/L, and heterotrophic denitrification using the soluble remaining COD present in the MBR inlet probably played a marginal role. Heterotrophic denitrification must have coexisted with other denitrification processes, such as methane denitrification and/or anammox. The low nitrite concentrations detected in the pre-anoxic compartment, and the negligible ammonium removal rate observed in this compartment, suggest that denitrification via anammox probably was not substantial but might still make up a fraction of the overall nitrogen elimination. This fact will be studied in detail by means of microbiological tools and batch activity tests in the sections "Microbiological analysis" and "Anaerobic batch experiments", respectively. Furthermore, methane denitrification via nitrate as described by Equation 20 and Equation 21 (Haroon et al., 2013) probably was one of the simultaneously operating denitrification mechanisms.

An NRR of 79 mgN/(L·d) in a similar UASB coupled in series to a pre-anoxic MBR system with different biomass carriers was reported (Sánchez et al., 2016). Due to the much higher averaged COD_T (261 mg COD/L) in the MBR post-treatment, a larger fraction of heterotrophic denitrification was assumed. A 1.2 L lab-scale membrane biofilm reactor was operated. In this work the methane was supplied from the inner

part of the membrane to the liquid phase where the biological reaction occurred. These authors obtained a surprisingly high NRR, up to 190 mgN/(L·d) (Shi et al., 2013). Another study from the same laboratory obtained an NRR of 684 mgN/(L·d). These remarkable values have not been replicated, even in the latest studies at lab scale or bench scale. For instance, a denitrification rate no larger than 40 mgN/(L·d) was observed using a newly discovered microorganism at lab scale (Bhattacharjee et al., 2016).

Taking this information into account, indications such as nitrogen removal that exceeds the predicted values for heterotrophic denitrification and a large rate of methane removal suggest that denitrification coupled to methane oxidation could be present in the system. At this point, there is still not enough information to assess whether AMO-D, DAMO, or a combination of both have been involved.

5.3.4. Influence of DO on methane and nitrogen mass balances

The large extent of the methane removal suggest that this compound acted as an electron donor for denitrifying a fraction of the eliminated nitrogen. Equation 20, Equation 21, Equation 22 reveal that nitrogen elimination could have proceeded through either or both of the denitrification coupled to methane oxidation processes explained above, AMO-D and/or DAMO. The intake of oxygen into the pre-anoxic compartment probably balanced the extension of either the aerobic or the anaerobic pathway.

MRR and NRR are depicted in Figure 5. 6 as a function of ORR. $k_L a^{O2}$ in the pre-anoxic compartment was 0.9 d^{-1}. To promote understanding of this figure, it is divided into three different stages depending on ORR (see stages I, II and III in Figure 5. 6). Experimental periods in which DO in the aerobic compartment diminished to values below 0.9 mg/L were assigned to stage I. The observed stable MRR values of 70-80 mgCH$_4$/(L·d) were only partially employed for denitrification since the degree of nitrogen removal in this period was low. A possible reason for this result might be that the presence of microorganisms related to the DAMO pathway were not present to a sufficiently large degree to perform their function since their doubling time has been estimated to be 1-2 weeks (Ettwig et al., 2010). The majority of these results were collected in the first 120 days of operation.
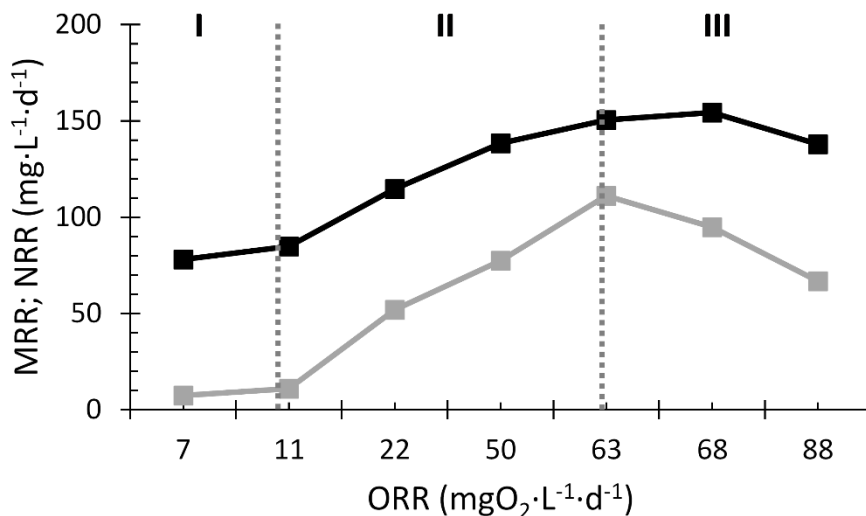
Figure 5. 6. Methane (■) and nitrogen (■) removal rates (MRR and NRR, respectively) as a function of the oxygen removal rate (ORR) in the pre-anoxic compartment.

In stage II, linear increases in both MRR and NRR as a function of ORR were detected, indicating that methane and nitrogen removal were correlated.

In stage III, a dramatic decrease in the NRR was detected. This result shows that, although anaerobic and aerobic denitrification coupled to methane oxidation can coexist, there is likely to be a threshold past which anaerobic processes and the overall denitrification rate diminish. Even, in this stage III, the remarkable ORR might partially decline the fraction of nitrogen elimination related to the heterotrophic processes. As a consequence, a slight decline in the MRR was detected, probably due to methane removal associated with DAMO processes. Nevertheless, the observed drop was much more gradual than in the NRR case, since AMO-D or other alternative processes might have been involved. Moreover, if the removed DO of over 90 mgO$_2$/(L·d) was completely available to the microorganisms, stoichiometric calculations only explain a maximum of 29% of the detected methane oxidation. As a consequence of these results, the coexistence of both AMO-D and DAMO pathways is suggested.

Even considering that TN was not fully eliminated, this study opens the door to the possibility of removing both dissolved methane and TN in anaerobically treated sewage. Up to 9.6 mg TN/L (via AMO-D) and 22.4 mg TN/L (via DAMO) might be removed considering a dissolved methane concentration of 16 mg/L, observed in the effluent of UASB systems treating sewage (Noyola et al., 2006), and Equation 20, Equation 21 and Equation 22, respectively. The maximum TN removed during the current study was 15 mg/L, with an average dissolved methane concentration of 29±4 mg/L. The previous results regarding the impact of DO on the process, indicated that both the aerobic and anaerobic pathways may coexist in the system. In this sense, encouraging the DAMO pathway offer an opportunity to increase the amount of TN denitrified with methane. Regarding phosphorus, not eliminated in the current study, it could be removed using a chemical precipitation process. Alternatively, there is the possibility of using nutrients contained in the treated permeate, as nitrogen and phosphorus sources, in a scenario in which the effluent of the MBR post-treatment system would be reused for crop irrigation, reducing chemical fertilizers application.

5.3.5. Microbiological assays

Experimental results suggest that both AMO-D and DAMO processes coexist, but proof is still lacking. To determine whether the representative microorganisms related to these processes were present and to estimate their importance, a microbiological study was conducted.

A FISH determination was carried out for detecting methanotrophs, typical methane oxidizers involved in AMO-D. Both, DAMO archaea and bacteria were searched for as well, since they should be present when the anaerobic pathway proceeds with either nitrate or nitrite as the electron acceptor. Anammox representatives were sought too since they can coexist with DAMO archaea and bacteria (Haroon et al., 2013) and both ammonium and nitrite were present in the pre-anoxic compartment.

Type I methanotrophs were detected in large quantities in both suspended and Levapor biomass with only one difference: the biomass present in the samples drained from Levapor carriers was aggregated in clusters, whereas the microorganisms collected as suspended biomass were more dispersed (Figure 5. 7). The substantial

observed presence of type I methanotrophs demonstrates the importance of the AMO-D pathway.
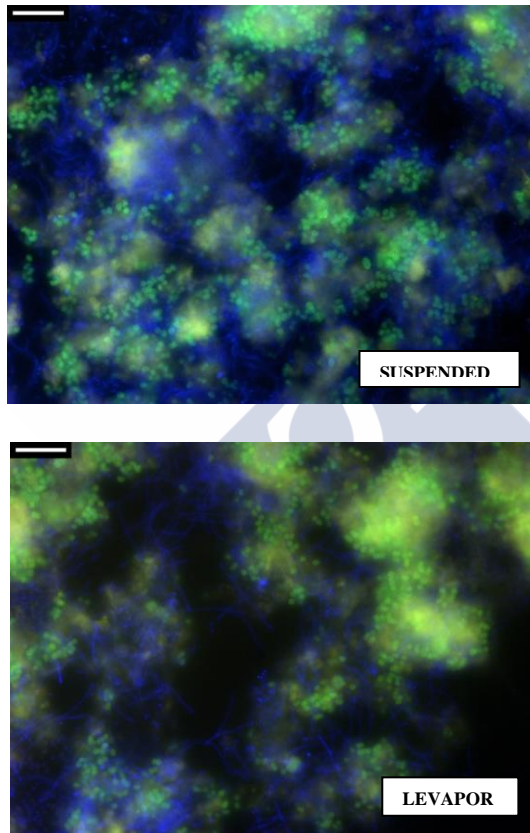

Figure 5. 7. Fluorescence in situ hybridization of biomass of the reactor during the day 215th of continuous operation. The hybridization probes were MA450 Cy3 (red) methanotrophs type II, MG 84 + MG705 (green) Fluos methanotrophs type I, DAPI (blue). Scale bar = 25 μm.
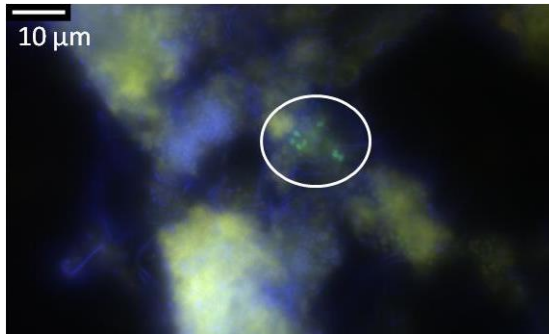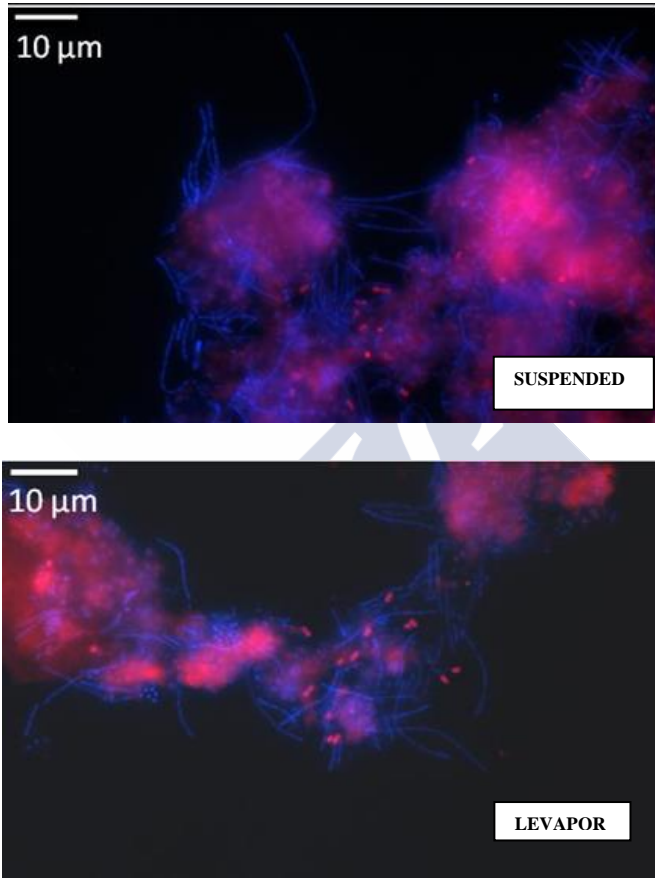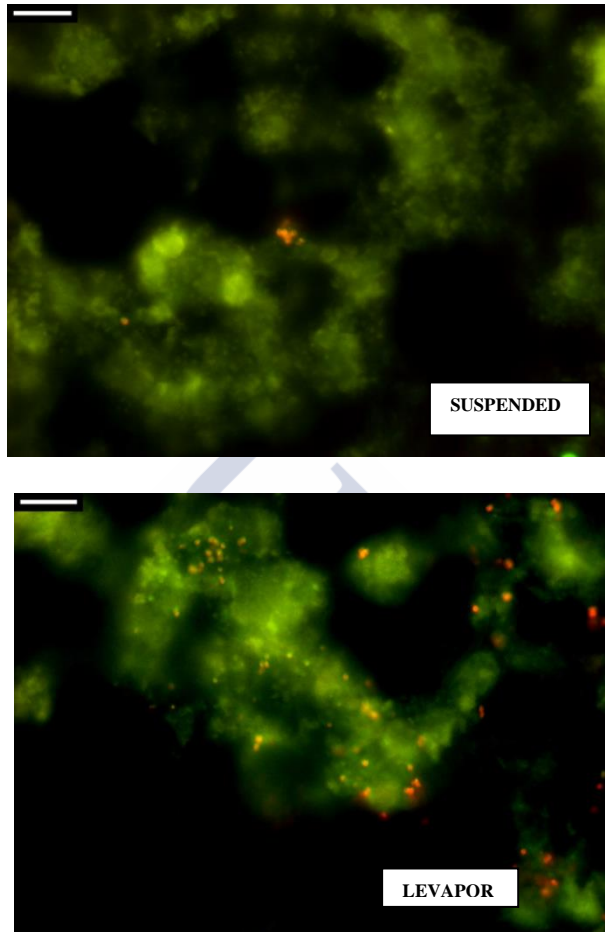
Figure 5. 8. Fluorescence in situ hybridization of biomass of the reactor during the day 215th of the continuous operation. The hybridization probes were DAMO archaea (DARCH872) Cy3 (green) and DAPI (blue). Scale bar = 10 µm

Additionally, the DAMO pathway was also studied. The consortium of DAMO and anammox has been demonstrated to be capable of carrying out anaerobic methane oxidation coupled to denitrification through reactions described in equations (5.2) to (5.4). Thus, the microorganisms involved in this mechanism were searched for. These DAMO microorganisms were detected, especially as bacteria (Figure 5. 9) in both suspended and as biofilm. Moreover, anammox positives have been detected mostly as biofilm (Fig. 5.10). The coexistence of both DAMO and anammox indicated the presence of the anaerobic consortium predicted by (Haroon et al., 2013) and confirmed the elimination of methane and nitrogen through DAMO via.

Figure 5. 9. Fluorescence in situ hybridization of biomass of the reactor during the day 215th of the continuous operation. The hybridization probes for DAMO bacteria were DBACTmix: (DBACT193 and DBACT1027 Cy3 (red)) and DAPI (blue). Scale bar = 10 μm.

Figure 5. 10. Fluorescence in situ hybridization of biomass of the reactor during the day 215th of the continuous operation. The hybridization probes for anammox bacteria were AMX820 Cy3 (red) and AMX368 Fluos (green). Scale bar = 10 μm.

DAMO bacteria was detected both in the biofilms and in the suspended solids samples. The presence of DAMO bacteria in the suspended solids sample was a non-expected result of this research, as this was subjected to alternating anoxic and aerobic conditions, due to the recirculation. These microorganisms are difficult to grow and could be hindered by the presence of dissolved oxygen. Methane oxidation, and especially denitrification was partly inhibited by oxygen, in a DAMO enriched culture

(Luesken et al., 2012). In this sense, the results of the present research demonstrated that DAMO could be present in alternating anoxic and aerobic environments.

5.3.6. Anaerobic batch experiments

Once DAMO positives had been microbiologically demonstrated by FISH tests, batch assays were performed to confirm its activity. Figure 5. 6 indicates that an increase in ORR affects NRR negatively, indicating that an excess of oxygen diminished the denitrification capacity of the system. Thus, denitrification might have taken place by both aerobic and anaerobic mechanisms. Batch anaerobic assays were carried out to confirm the possibility of DAMO activity in the pre-anoxic compartment. The assays conducted are summarized in Table 5. 2. The main results of the discontinuous assays are depicted in Figure 5. 11.
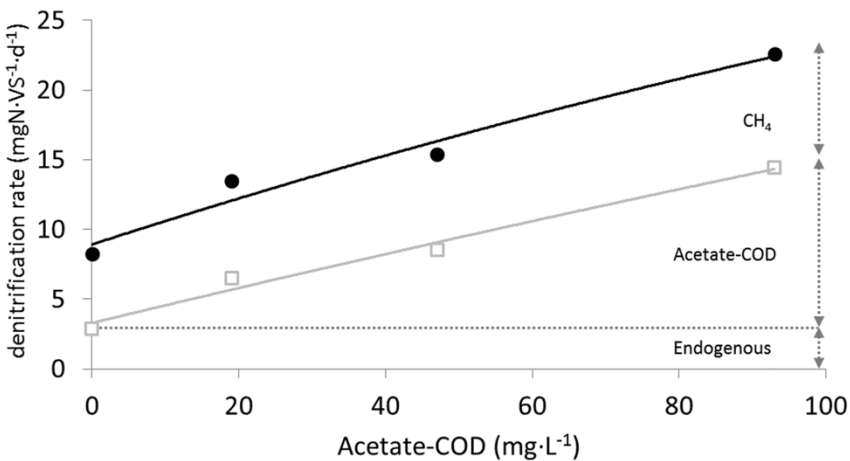
Figure 5. 11. Denitrification rates obtained by means of batch assays with biomass collected from the anoxic compartment of the continuous system. –Case studies: I) Basal activity: neither acetate nor methane were present (indicated on the Figure with a dotted line). II) Basal + acetate-COD (□): acetate was externally added. III) Basal + acetate-COD + Methane (●): acetate and methane were added as electron sources.

To estimate the basal denitrification, case study I was performed using a flask with no externally added electron donors. Neither acetate nor methane was present. Considering that the biomass concentration was approximately 3.9 gVS/L (including

that suspended and attached to the biofilm carriers) and was equal in all flasks, the observed absolute rate of 4.1 mgN/(gVS·d) was considered to be the basal denitrification, which was equal in all three case studies.

When only acetate COD was added in case study II, the higher acetate concentration was, the higher NRR became. The maximum observed value was 15 mgN/(gVS·d).

When both methane and acetate were used as an electron donor (case study III), the maximum observed value of 23 mgN/(gVS·d) was detected. An increase of 4-5 mgN/(gVS·d), corresponding to 16-20 mgN/(L·d), was observed, regardless of the acetate COD concentration in the bottle. This activity was caused by the DAMO-related microbial populations detected by FISH (see Figure 5. 7 and Figure 5. 8), since anaerobic conditions were present in the bottles. The observed values resulted from a combination of three different electron donors: basal, acetate COD and methane. The coexistence of this set of electron sources led to an increase of the denitrification capacity through the coupling of different carbon sources. Consequently, several different mechanisms, such as endogenous, heterotrophic and DAMO, were active.

These results confirm the presence of anaerobic DAMO denitrification in the pre-anoxic MBR pointed by FISH analysis, since the biomass employed for carrying out the experiments was collected directly from the system and no oxygen was present. Because nitrate was the only oxidized nitrogen species present, DAMO archaea were probably responsible for the first stage of DAMO denitrification (Haroon et al., 2013).

An activity assay with biomass taken from a *Candidatus Methylomirabilis oxyfera* enrichment in the absence of any other source of organic matter and using nitrite as an electron acceptor was previously published. Even in the optimal experimental scenario at a pH of 7.5, without salinity indicators and at 35 °C, the highest observed activities were 1.51 mgN/(gVSS·d) (He et al., 2015) lower than the observed values in our batch assays when neither acetate COD nor methane was present. In the study with the enriched culture, it was suggested that the excreted products of DAMO were employed as a carbon source by heterotrophic denitrifiers. This assumption is consistent with the results observed in the present research. An NRR of 40 mgN/(L·d) (Bhattacharjee et al., 2016) was obtained from a pure culture DAMO reactor operating under anaerobic conditions, higher than the value of 19 mgN/(L·d) obtained in the

current study but still of the same order. The experimental values obtained in the current study are in accordance with those observed in a previous work (Sánchez et al., 2016), where other types of wastewater and carrier were present. Nevertheless, in other research, batch assays at 33 °C using nitrate as an electron acceptor were performed. In this case, the successful experiments were conducted in the presence of oxygen by means of the AMO-D mechanism, instead of DAMO, which was acting in the previous two experiments. In this aerobic case, a remarkably high NRR of 600 mgN/(gVSS·d) was observed when methane and up to 90 mbar of DO were present.

Thus, the highest observed value was an order of magnitude lower than the typical values of 250 mgN/(gVSS·d) observed in a CAS system when the SRT has been set up properly (Metcalf&Eddy et al., 2014). Moreover, the stoichiometry described by Equation 21 shows a higher theoretical denitrification potential for the DAMO mechanism. Thus, further investigation should be conducted to increase the denitrification rates to values that are able to compete with traditional systems that consume much more energy.

On the other hand, anammox activity has not been detected by means of the protocol published in (Dapena-Mora et al., 2007). Despite of being detected by FISH analysis, batch assays demonstrated that the role anammox has been negligible in comparison with other co-existing denitrification processes.

5.4. Conclusions

The newly presented pre-anoxic MBR post-treatment is capable of consistently removing 80% of dissolved methane with peaks up to 95%.

Up to 10-15 mgN/L of the wastewater was continuously eliminated.

Recycle ratio and dissolved oxygen played a crucial role to govern the denitrification mechanisms.

Microbiological studies elucidated that AMO-D was present due to the great abundance of the most representative microorganisms.

Batch assays and microbiological tests undoubtedly demonstrated the presence of DAMO activity.

Mass balances indicated that the fate of a fraction of the eliminated methane was still unknown; therefore, further studies are required to reveal its destination.

Chapter 6

Overview of the competition among oxidizers of methane and ammonium in an oxygen scarcity environment[3]

Summary

Ammonium and methane oxidizers coexist in both natural ecosystems and WWTPs. Scarce information about their interactions is already available. In this chapter, many strategies for enriching and growing their representatives in the lab were conducted. Subsequently, the presence of ammonium and methane in pure cultures of *Nitrosomonas europaea* and *Methylomonas lenta* was tested. *Nitrosomonas europaea*'s oxidized both ammonium and methane if the second in headspace was 5% and inhibition was observed if methane was larger than 5%. Ammonium on *Methylomonas lenta* inhibited the activity in every concentration tested. The impact of nitrite (ammonium oxidizers' product) on *Methylomonas lenta* indicated an enhancer effect (up to 239 µM) or an inhibitor role (from 239 to 1020 µM) depending on the nitrite concentration. This study opens the door to design a continuous competition experiment with acceptable concentrations for both substrates when *Nitrosomonas europaea* and *Methylomonas lenta* are present.

6.1.   Introduction

Leachate of fertilizers, as long as combustion of forests biomass and fossil fuel have been considered as huge human sources of reactive nitrogen which caused tremendous impacts on nitrogen balance. With data updated on 2000, the first releases about 100 Tg/year mostly as ammonium, and the combustion processes over 60 Tg/year majorly as NO_X (NO, NO_2, N_2O, among others) (Fields, 2004). The steady increase in nitrogen concentration in the natural ecosystems has been observed, especially in environments near to an urban area, affecting aquatic, air and soil ecosystems. These high nitrogen ions releases are also responsible for N_2O emissions (Castro-Barros et al., 2016). N_2O has been considered a strong greenhouse gas (GHG), with an associated global warming potential (GWP) up to 298 (respect to CO_2) (https://www.epa.gov/ghgemissions/overview-greenhouse-gases, last access July 2018).

Wastewater streams have been also considered one of the largest sources of nitrogen responsible for unbalances in the natural water bodies. A massive presence of nitrogen compounds mostly ammonium in water medium, coming from urine discharged in the wastewater and washout of fertilizers, may unbalance the DO concentration in the water medium. A typical example has been eutrophication. The presence of large concentrations of the fully oxidized nitrogen anion nitrate has been one of the main actors in this phenomenon (Wilkinson, 2017). Algae blooming may destabilize both DO availability for the coexistent species and the light irradiation into the ecosystem. Thus, wastewater containing nitrogen should be partially treated before returning to nature. To prevent this, nitrogen compounds have been regulated and included in the group of the main pollutants to remove in the wastewater treatment plants (WWTP) (Metcalf&Eddy et al., 2014). Figure 6. 1 summarizes the nitrogen cycle transformations which normally happen in these facilities.
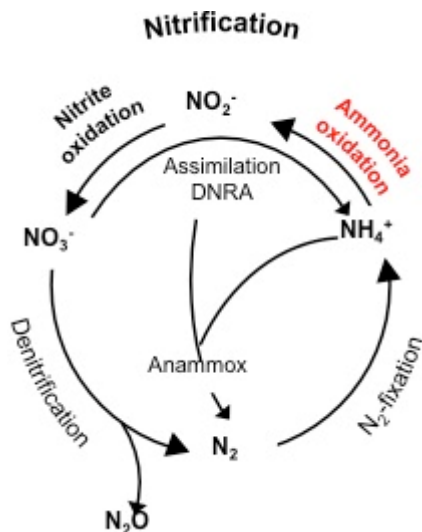
Figure 6. 1. N-cycle and transformations typically conducted in a full-scale wastewater treatment plant. Source: www.microbial-ecology.net.

Figure 6. 1 contains the most important biological transformations which can be carried out in a WWTP. In a conventional activated sludge treatment (CAS), the most spread wastewater treatment worldwide, normally heterotrophic pre-anoxic denitrification systems are present. Thus, nitrogen mostly present as ammonium in the raw wastewater is oxidized into nitrite (by means of ammonium oxidizing bacteria (AOB hereafter). Ammonium transformation is a two-steps process performed firstly to nitrite and subsequently oxidized to nitrate by nitrite oxidizing bacteria (NOB). Ammonium oxidation to nitrite has been typically performed by bacteria such *Nitrosomonas*, *Nitrosospira* or *Nitrosococcus* in its specific optimal conditions, depending even on the especifically involved species. The presence of the enzyme ammonium monooxygenase (AMO) encoded by the gene *amoA* has been considered an essential requirement for carrying out the ammonium oxidation.

Heterotrophic populations are in charge of employing the external organic carbon contained in the wastewater for reducing the nitrogen anions to nitrogen gas. Both organic carbon and nitrogen removal processes have been considered large energy demanding processes and converted the WWTP in large energy consumers. Nowadays, a new challenge for achieving a self-sufficient energy WWTP in the next

few years (Gu et al., 2017) has been recently appeared. In the denitrification process, especially if it is not carried out in all its extension, is possible the N_2O generation.

In the 90´s the anaerobic ammonium oxidation (anammox) process was discovered in a pilot plant in the Netherlands (Kartal et al., 2010; Mulder et al., 1995). Anammox is an autotrophic process. External organic carbon is not required so organic carbon in the wastewater can be used for other purposes such as obtaining biogas in an anaerobic treatment, instead of denitrifying. The Equation 31(Jetten et al., 1997) summarizes the biochemical transformations of anammox which are graphically depicted in Figure 6. 1.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

Equation 31

Traditionally, the transformation of nitrite into nitrate proceeded kinetically much faster than ammonium oxidation, hindering the accumulation of nitrite. As a consequence, intercepting nitrogen oxidation in nitrite as intermediate has been a huge bottleneck when developing and scaling-up new technologies based on anammox processes. Moreover, the typical doubling time of AOB has been detected slightly higher than NOB triggering the tendency of the fully ammonium oxidation into nitrate instead of being intercepted in the desired nitrite (De Clippeleir et al., 2013). For these reasons, technological solutions based on anammox should face the struggle of stopping ammonium oxidation in nitrite.

The contrary conditions of ammonium oxidation (oxygen-demanding) and anammox (strict anaerobic environment) (Dongen et al., 2001) required a technological solution able to fill these requirements. Traditionally, anammox process required more than one reactor like in the SHARON process. In the first reactor, partial ammonium oxidation occurs which produces nitrate. In the second reactor, which is anoxic, the produced nitrite and the leftover nitrate are converted into dinitrogen gas by anammox. Very recently, granular systems creating aerobic, anoxic and anaerobic environments depending on the depth of the layers have been developed. Hence, the anammox process is currently used even at full scale. It can be fed with the rejection water from anaerobically digesting the sludge (Morales et al., 2015) or in waterline. The anammox process has been even demonstrated in mainstream at lab scale even at 10 ºC (Reino

et al., 2016) and these systems could be upscaled to industrial sizes in a short period of time.

Partial nitrification in combination with anammox systems can be considered as a promising alternative to upgrade the nitrogen removal cycle at full scale with remarkable energy savings. Recent mainstream related discoveries and energy savings from 4.0 down to 0.8 kWh/kgN will promote the quick growth of these nitrogen removal treatments (Lackner et al., 2014). Full-scale nitrogen elimination facilities based on partial nitrification will undoubtedly grow in the next years.

Traditionally, it was reported that ammonium oxidation has to proceed in two steps. Ammonium is first oxidized to nitrite by ammonium oxidizing bacteria or archaea and the produced nitrite is subsequently oxidized by nitrite oxidizing bacteria to nitrate. However, in the last years it was demonstrated that some *Nitrospira* are able to carry out complete ammonium oxidation (comammox) to nitrate by one single microorganism (van Kessel et al., 2015) as predicted by Costa et al., (2006). This is a novel process which may better explain the actual abundance distribution of the ammonium and nitrite oxidizing microorganisms in nature and in a WWTP. Still, there are no evidence of the implications of this discovery but it is possible that any changes in the nitrogen cycle are expectable, not only as depicted in Figure 6. 1, also other modifications might appear.

Methane sources as a consequence of human activity account up to 70% of the total annual emission (https://icp.giss.nasa.gov/education/methane/intro/cycle.html, last access July 2018). Methane is a greenhouse gas (GHG) with a warming potential of 34-CO_2 equivalents for a 100 year time horizon (Myhre et al., 2013). Wetlands, energy production, and enteric methanogenic fermentations have been the major methane sources worldwide. Furthermore, this gas has been often emitted during the wastewater treatment, accounting up to 5% of the total methane releases (Augenbraun et al., 2016).To prevent its non-controlled emission into the atmosphere, dissolved methane should be transformed/removed within the WWTP facility. This compound appears when strong anaerobic conditions are present and organic carbon is broken down in a reductive ambient. Both, organic matter and anaerobic conditions have been found in some units of WWTPs. Apart from the anaerobic methanogenic reactors, methane can appear in sludge thickeners, buffer storage tanks and even in sewers

(Daelman et al., 2012). In these latter units and in many other places, either in nature or in a WWTP, methane, and ammonium have to necessarily coexist.

The simultaneous presence of methane, ammonium and other nitrogen ions could exert an impact on those microorganisms using exclusively one of the compounds as substrate. Many studies pointed out the biochemical interactions among not only ammonium and methane (Zheng et al., 2014) but also with other nitrogen anions. Still, these connections have not been widely explained. Enhancements, inhibitions and nitrogen/methane concentrations should be further studied to predict the behavior when methane, ammonium, and nitrite oxidizing microorganisms are coexisting in the same ambient.

Microbiological processes conducted in WWTPs are a reflect of the essential processes occurred in nature. Variables such as pH, dissolved oxygen (DO), optimal substrate concentrations or potential inhibitors should be thoroughly studied before setting up a process at full scale. In a similar way, in nature and marine ecosystems, for example, not only nitrogen species are present, also carbon compounds such as methane appear since the organic carbon break down takes place in the existing anaerobic environments. The oxidation of both compounds has been an exothermic process. As a consequence, microorganisms such ammonium and methane oxidizers obtain their energy requirements by means of the thermodynamically favorable biochemical processes. Thus, ammonium and methane-oxidizing bacteria (hereafter AOB, MOB, respectively) may coexist in those environments where both substrates are present.

Although AOB and MOB have been widely studied individually, their interactions are still unknown. An environment where oxygen is barely present is a requirement for the existence of methane and both ammonium and methane oxidation require the presence of oxygen (micro-aerobic) for being conducted. The study of their interrelations when oxygen is limited will provide valuable information for knowing further about the oxidation processes in these types of environment present in nature and in the WWTP. The enzyme methane monooxygenase (MMO) has to be present for conducting methane oxidation. Its alpha subunit is encoded by the *pmoA* gene. This *pmoA* gene has been detected relatively similar in many different organisms. As a consequence, it is normally employed as a marker gene to detect methanotrophs.

Genes and proteins encoding AMO and MMO (*amoA* and *pmoA*, respectively) share high sequence identity despite their different physiological roles (Holmes et al., 1995) since they might have shared a common ancestor. Evidence of the concurrence of both AOB and MOB representatives could be found in volcanic (Daebeler et al., 2014) or cultivation soils (Zheng et al., 2014) as long as aquatic landfills such as lakes (Junier et al., 2010) or marine environments. *Methylosarcina*, *Methylobacter,* and *Methylomonas* were the most common representative genus of aerobic methanotrophs. Since AMO and MMO have similar sequences, it was reported that AOB have been able to catalyze methane oxidation (Hyman and Wood, 1983) into methanol and MOB have been capable of oxidizing ammonium (Holmes et al., 1995) into nitrite. From the spatial point of view, ammonium and methane molecules are similar and both can be described with an orbital hybridization model sp^3. The substrate of MOB (methane) can be an inhibitory compound for ammonium oxidation and vice versa (Holmes et al., 1995; Nyerges and Stein, 2009; Suzuki et al., 1976) as extensively reported. Moreover, the oxidation products of both enzymes may be inhibitory and toxic for some microorganisms in charge of carrying out biochemical processes (Stein et al., 2012). Methanol and hydroxylamine are examples of toxic and irreversible effects on MOB and AOB (Hanson and Hanson, 1996), respectively. Nitrite toxicity has to be investigated for both AOB and MOB but its presence might play its role as an inhibitor for MOB (King and Schnell, 1994). Hence, an intensive study of ammonium oxidizing activities in presence of methane and methane oxidation in presence of ammonium and nitrite should be conducted in order to explore and understand the interactions among AOB and MOB. When dealing with oxidizers, the minimum dissolved oxygen (DO) required for starting the oxidizing activity should be found to verify the minimum DO threshold to be established in the final experiment. Nevertheless, these specific effects and thresholds depended on the involved genera/species.

In this study, continuous and discontinuous strategies for obtaining AOB and MOB enriched and pure cultures have been tested. Their detection via Fluorescence In Situ Hybridization (FISH) and identification by means of microbiological tools such as Polymerase Chain Reaction (PCR) and the subsequent cloning and sequencing of the obtained products were performed.

For obtaining further information about AOB and MOB interactions, approaches for determining:

I) The inhibitory capacity of methane (MOB substrate) in the oxidizing activity of AOB,

II) the inhibitory capacity of ammonium (AOB substrate) in the oxidizing activity of MOB,

III) The inhibitory capacity of nitrite (product of AOB metabolism) in the oxidizing activity of MOB;

were carried out. Moreover, the minimum DO requirements for the AOB and MOB representative microorganisms have been found.

The results of this study were carried out to design a further competition experiment out of the scope of this Chapter 6.

6.2. Materials and methods

6.2.1. Enrichment continuous culture reactors

A system composed by two coupled in series continuous stirred tank reactors (CSTR) (Figure 6. 2) (2 L each) was operated for 170 days.

Both vessels were built with a combination of glass and stainless steel (Applikon Biotechnology BV, Schiedam, The Netherlands) equipped with mechanical stirrers. DO and pH probes (Applikon Biotechnology BV, Schiedam, The Netherlands) were installed and membranes were setup to prevent biomass washout. In order to control the temperature, a heating jacket was built over the reactor being the heating medium tap water. The first, A reactor, was inoculated with a mixed culture. It was operated in conditions able to promote the growth of ammonium-oxidizing microorganisms.
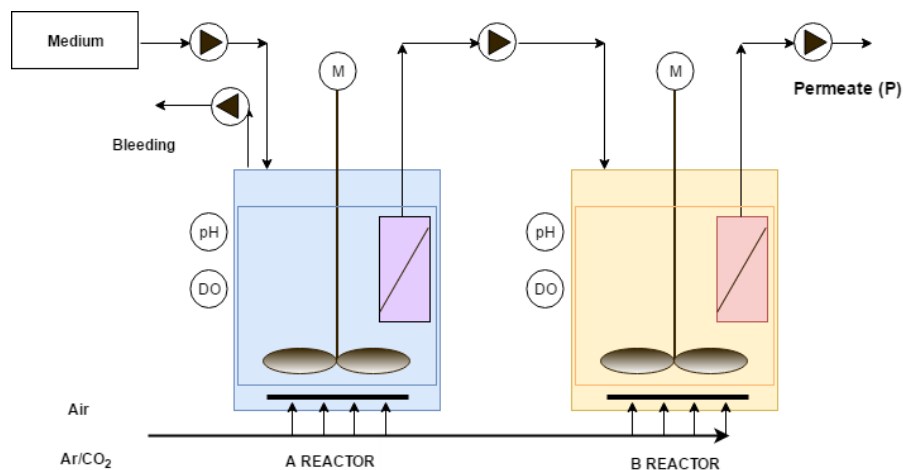
Figure 6. 2. Experimental setup composed by two coupled in series continuous stirred tank reactors. The first, A reactor, aimed at converting ammonium into nitrite. The second, B reactor, intended to fully nitrify both nitrite and ammonium into nitrate.

Ammonium was supplied and oxygen concentration was low. The gas line was composed of two different streams linked before entering the system. Through one of them, air was provided. Furthermore, a dilution stream was setup with a mixture of 95%/5% Ar/CO_2, for achieving a final concentration of oxygen in the incoming gas stream of 2%. The gas stream was intentionally water saturated before entering the reactor to prevent the liquid level decrease by means of a no controlled evaporation of the liquid reaction phase. A pH control system by adding $KHCO_3$ (1 M) was set up for maintaining this variable in the fixed range. For correctly manipulating the average time the microorganisms stay in the system, the solids retention time (SRT), a withdrawal stream was set up. In the second, B reactor, a similar setup was present. Alternatively, the set operational conditions aimed at promoting NOB microorganisms. The gas flow rate was established to obtain a DO concentration around the saturation to guarantee the microorganisms' demands. Finally, this value remained at 30% which led to an environment of oxygen excess. The entire system was fed with an inlet flow of 2 L/day with a synthetic medium composed of 1000 μM NH_4Cl, and 1000 μM $KHCO_3$ and trace elements. The medium was always prepared by autoclavation before entering the system to remove the presence of external microorganisms. The system was sampled

and analyzed twice a week and its tracking was carried out every day by employing NO_2^-/NO_3^- stripes.

The most important parameters when designing the operation in the A reactor were SRT and temperature. The calculation of SRT has been conducted with the Equation 32.

$$SRT = \frac{X_i \cdot V_i}{Q_X \cdot X_x}$$

Equation 32

where:

$X_i \equiv$ mixed liquor volatile suspended solids (MLVSS) in each reactor,
$V_i \equiv$ individual reactor volume,
$Q_x \equiv$ excess of biosolids purged,
$X_x \equiv$ MVLSS in the purged stream.

Table 6. 1 summarizes the set SRT and temperature values during the experimental stages.

Table 6. 1. Operational strategy in the A reactor

Stage	Day of operation	SRT(*) (days)	Purged flow (mL/day)	Temperature (ºC)
I	0-19	Not purged	0	20-23
II	20-32	18	110	20-23
III	33-64	13	150	20-23
IV	64-69	13	150	31
V	70-76	5	400	31
VI	77-168	3	667	31

(*) SRT is referred to A reactor

6.2.2. Biological oxygen monitor (BOM) assays

Biological oxygen monitor tests were carried out in order to determine the minimum oxygen threshold from which AOB and MOB representatives are sensitives.

A 2.5 mL cell container was filled with the respective substrate for each microorganism and oxygen. Listed below are the specific concentrations added in each experiment. The respective substrates, ammonium, and methane, were added in a 30% excess to the oxygen saturated medium:

1) AOB. 20 µL of 2 mM NH_4Cl and the rest of 2.5 mL filled with DMSZ without ammonium medium. The medium was oxygen saturated by blowing air.

2) MOB. 1.5 mL *Methylomonas lenta* pure culture medium oxygen saturated by blowing air. 1 mL of methane saturated liquid stock solution was added.

Dissolved oxygen concentration was monitored with the probe Unisense RC 300-350 which automatically registered the experimental data. Sensitivity 1 µmol O_2/L.

6.2.3. Specific analytical methods

Ammonium determination protocol (OPA method, spectrofluorophotometer, CARY Eclipse)

Ammonium was measured colorimetrically using a modified orthophataldialdehyde (OPA) assay (Taylor et al., 1974) and nitrite was measured by the salicylic acid (Griess) reaction. This method is recommended for a wide range of measurements, from sub-micromolar to concentrations upper than > 100 µM NH_4^+. This method releases less harmful species than others like indophenol blue method.
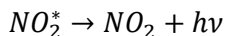
After the OPA addition, a product which can be quantified is formed. Its concentration can be measured by fluorescence at a wavelength of 420 nm (Holmes et al., 1999).

Nitrite/nitrate determination with the nitric oxide analyzer (NOA280i)

The 280i Nitric Oxide Analyzer (NOA) is a high-sensitivity detector for measuring nitric oxide based on a gas-phase chemiluminescent reaction between nitric oxide and ozone. The following set of chemical reactions are the basis of the principle of measurement.

$$2NO_3^- + 3V^+ + 2H_2O \rightarrow 2NO + 3VO_2^+ + 4H^+$$

$$NO + O_3 \rightarrow NO_2^* + O_2 \hspace{3cm} \textbf{Equation 33}$$

$$NO_2^* \rightarrow NO_2 + h\nu$$
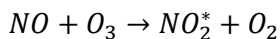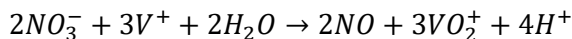
The sensitivity of this method was remarkably high into the range 0.5 of 24 µM of nitrate.

6.2.4.   Discontinuous enrichment culture reactors

Nitrosomonas europaea as ammonium oxidizing bacteria (AOB)

A pure culture of _Nitrosomonas europaea_ was run in the laboratory of the Department of Microbiology of The Radboud Universiteit Nijmegen. The inoculated pure culture had been stored frozen at -80ºC. The culture had been previously harvested before the beginning of the internship. The inoculum was growth for obtaining a medium with a proper concentration of ammonium oxidizing bacteria. The culture medium Deutsche Managementsystem Zertifizierungsgesellschaft (DMSZ) was purchased to the _Leibniz Institute Sammlung von Mikroorganismen und Zellculturen GmbH_ of Germany. The actual composition (Table 6. 2) was downloaded at https://www.dsmz.de/home.html.

In order to correctly perform the inoculation of the culture bottles the following protocol was carried out:

I) 20 mL of medium prepared as previously described was added into four different serum bottles of 100 mL each. II) The headspace was not further treated due to the interest of the oxygen/air availability in the headspace. III) These bottles were perfectly capped with rubber stoppers. IV) All bottles were autoclaved at 120ºC for 20 min to remove any no desirable microorganisms which might be present in the laboratory atmosphere before adding the microorganisms. V) Once the bottles were autoclaved and cooled, 1 mL of pure _Nitrosomonas europaea_ were carefully added in each bottle by using sterilized disposable lab materials in a sterile atmosphere.

Table 6. 2. Culture medium employed for the Nitrosomonas europaea enrichment

DMSZ *Nitrosomonas europaea*

Compound	Concentration ($mg \cdot L^{-1}$)
NH_4Cl	535
KH_2PO_4	54
KCl	74
$MgSO_4 \cdot 7H_2O$	49
$CaCl_2 \cdot 2H_2O$	147
NaCl	584
Trace elements	1 mL
Phenol red (pH indicator)	10mg/50mL
$KHCO_3$	1 M
HEPES (*)	4,800

(*) HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

The bottles were cultivated in the dark in a shaker at 30 rpm during approximately 15 days. Ammonium and nitrite were monitored every day by employing the respective stripes. pH was maintained at 7-8 as recommended by Engel and Alexander, (1958). This parameter was double checked with both the stripes and by using the pH indicator phenol red.

Methylomonas lenta as methane-oxidizing bacteria (MOB)

Similarly to *Nitrosomonas europaea* (AOB), one of the representatives of methane oxidizing bacteria (MOB) *Methylomonas lenta* have been cultured. This inoculum was obtained from Gent Universiteit (Belgium) where they had a culture collection (BCCM/LMG). The protocol employed in the case of these microorganisms was in basis similar than the employed for AOB with some differences which are going to be described below. In Figure 6. 3 the recipe of the culture medium is described.

Table 6. 3. Culture medium employed for the Methylomonas lenta enrichment.

Stock	mL	Phosphate stocks	g·L^{-1}
50 x dNMS (*)	20	I	
Phosphate stock I	5	KH_2PO_4	10
Phosphate stock II	5	II	
Trace elements I	0.1	$Na_2HPO_4 \cdot 12H_2O$	71.7
Trace elements II	0.5	$NaH_2PO_4 \cdot 2H_2O$	12.58
VI	77-168		

(*) dNMS recipe collected from (Hanson et al., 1991)

The protocol for preparing the culture bottles was similar than the above detailed for the AOB microorganisms. The only differences in the current case were the culture medium and the steps II), III) and V) of the AOB culture preparation protocol. The remaining steps were conducted exactly than in the previous AOB culture protocol. The adaptations to MOB cultures were as follows:

II) The bottles were perfectly capped with rubber stoppers and pure methane was injected through the stoppers in the same volume than that of air present in the headspace. III) The overpressure was released with a needle of a syringe, obtaining with this method a perfect mixture of 50% CH_4 and 50% air (v/v). V) Once the bottles were autoclaved and cooled, 1 mL of pure *Methylomonas lenta* were carefully added in each bottle by using sterilized disposable lab materials in a sterile atmosphere.

The bottles were cultivated in the dark in a shaker at 30 rpm during approximately 15 days. The tracking of the culture was double checked. On the one hand, to eye due to the characteristic large flocs formed by this microorganism and by analyzing the methane declining concentration of the headspace by means of the gas chromatograph Agilent series 6890 equipped with Porapak Q and sieve columns.

6.2.5. *Nitrosomonas europaea:* ammonium oxidizing activity inhibition by methane

Four methane concentration levels (0, 5, 10 and 20% of CH_4) in the headspace were evaluated to study the inhibitory effect of this compound in the ammonium oxidation activity of *Nitrosomonas europaea*. For this study, 8 serum bottles of 120 mL each were filled with 30 mL of medium (Table 6. 2) with the only modification of the ammonium chloride concentration established at 1000 µM NH_4^+ in all bottles at the beginning of the experiment. Every assay has been performed in duplicate.

In order to correctly prepare the discontinuous reaction bottles the following protocol was performed in all bottles:

I) 30 mL of medium prepared as previously described was added into eight different serum bottles of 120 mL each. II) The bottles were perfectly capped with Wheaton stoppers and pure methane was injected through the stoppers by removing the equivalent volume of headspace (initially only air) and adding the equivalent volume of pure methane. An extra 1 mL of air was injected in all bottles to obtain a little overpressure and to ensure the correct methane headspace analysis. III) All bottles were autoclaved at 120ºC for 20 min to remove any no desirable microorganisms which might be present in the atmosphere before adding the microorganisms. IV) Once the bottles were autoclaved and cooled, 2 mL of pure *Nitrosomonas europaea* were carefully added in each bottle by using sterilized disposable lab materials in a sterile atmosphere. V) The experiment was fully carried out in the dark and in a shaker table set at 75 rpm.

When AOB were added, the experiment was considered started and liquid and gas samples were collected each 45 min during 5-6 hours. The methane concentration of the gas phase was directly measured by injection in the gas chromatograph. The liquid phase was centrifuged at 5000 rpm for 2 min to separate biomass and liquid phase. The supernatant was stored on ice until the end of the whole experiment. Later, both ammonium and nitrite were measured in all samples.

6.2.6. *Methylomonas lenta:* methane-oxidizing activity inhibition by ammonium

Four ammonium concentration levels (0, 100, 250 and 500 μM NH_4^+) in the liquid phase were evaluated to study the inhibitory effect of this compound in the methane oxidation activity of *Methylomonas lenta*. The initial methane concentration in the headspace was of 1% in all assays. For this study, 12 serum bottles of 100 mL each were filled with 20 mL of dNMS medium (Table 6. 3) with the only modification of the addition of ammonium chloride for obtaining the above-indicated concentrations of ammonium at the beginning of the experiment. Every assay has been performed in triplicate.

In order to correctly prepare the discontinuous reaction bottles the following protocol was performed in all bottles:

I) 20 mL of medium prepared as previously described was added into 12 different serum bottles of 120 mL each with the correspondent ammonium concentration. II) The bottles were perfectly capped with Wheaton stoppers and pure methane was injected through the stoppers by removing the equivalent volume of headspace (initially only air) and adding the equivalent volume of pure methane. An extra 1 mL of air was injected in all bottles to obtain a little overpressure and to ensure the correct methane headspace analysis. III) All bottles were autoclaved at 120ºC to remove any no desirable microorganisms which might be present in the atmosphere before adding the MOB. IV) Once the bottles were autoclaved and cooled, 4 mL of pure *Methylomonas lenta* were carefully added in each bottle by using sterilized disposable lab materials in a sterile atmosphere. V) The experiment was fully carried out in the dark and in a shaker table set at 75 rpm.

When MOB where added, the experiment was considered started and liquid and gas samples were collected each 45 min during 5-6 hours. The methane concentration of the gas phase was directly measured by injection in the gas chromatograph. The liquid phase was centrifuged at 5000 rpm for 2 min to separate biomass and liquid phase. The supernatant was stored on ice until the end of the experiment. Later, ammonium was measured in all samples.

6.2.7. *Methylomonas lenta:* methane-oxidizing activity inhibition by nitrite

Four nitrite concentration levels (0, 100, 250, 500 and 1000 μM NO_2^-) in the liquid phase were evaluated to study the inhibitory effect of nitrite in the methane oxidation activity of *Methylomonas lenta*. The initial methane concentration in the headspace was of 1% in all assays. For this study, 10 serum bottles of 100 mL each were filled with 20 mL of dNMS medium (Table 6. 3) with the only modification of the addition of sodium nitrite for obtaining the above-indicated concentrations of nitrite at the beginning of the experiment. Every assay has been performed in duplicate.

In order to correctly prepare the discontinuous reaction bottles the following protocol was performed in all bottles:

I) 20 mL of medium prepared as previously described was added into ten different serum bottles of 100 mL each with the appropriate nitrite concentration. II) The bottles were perfectly capped with Wheaton stoppers and pure methane was injected through the stoppers by removing the equivalent volume of headspace (initially only air) and adding the equivalent volume of pure methane. An extra 1 mL of air was injected in all bottles to obtain a little overpressure and to ensure the correct methane headspace analysis. III) All bottles were autoclaved at 120 ºC for 20 min to remove any no desirable microorganisms which might be present in the atmosphere before adding the microorganisms. IV) Once the bottles were autoclaved and cooled, 1 mL of pure *Methylomonas lenta* were carefully added in each bottle by using sterilized disposable lab materials in a sterile atmosphere. V) The experiment was fully carried out in the dark and in a shaker table set at 75 rpm.

When MOB were added, the experiment was considered started and liquid and gas samples were collected each 45 min during 5-6 hours. The methane concentration of the gas phase was directly measured by injection in the gas chromatograph. The liquid phase was centrifuged at 5000 rpm for 2 min to separate biomass and liquid phase. The supernatant was stored on ice until the end of the experiment. Later, nitrite was measured in all samples.

6.3. Results and discussion

6.3.1. Continuous enrichment culture reactors

The average ammonium concentration in the influent was of 1023±271 μM and neither nitrite nor nitrate were detected. The feeding entered the system at room temperature, ranged into 20-22 °C.

Reactor A

Reactor A was operated during 168 days aiming at enriching the medium in AOB populations. HRT was always maintained at 1 d. In Figure 6. 3 is shown the ammonium inlet and outlet as well as the accumulation of nitrite in the AOB reactor.
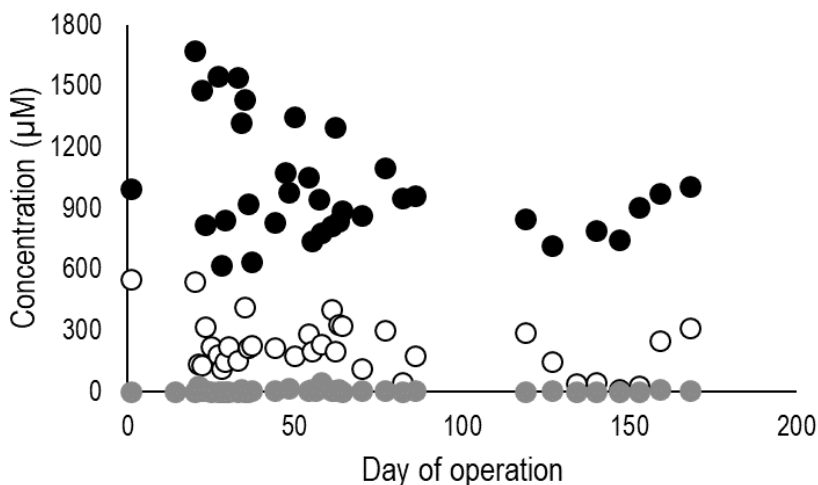

Figure 6. 3. Ammonium oxidation in Reactor A. (●) Ammonium in the medium; (○) ammonium and (●) nitrite in Reactor A

The system was able to counteract the initial variability of the influent since ammonium concentration in the outlet of Reactor A was very stable, always below 400 μM.

Ammonium was partially oxidized in a range of between 20 and 50%. Previous studies indicated that a decrease in the SRT enhanced the AOB/NOB ratio in the reactor (Guoqiang Wang, 2014; Kuo et al., 2006) with a nitrite accumulation detected in the

system. During the periods II to VII (Table 6. 1), SRT was decreased from 18 to 2 days by increasing the withdrawal flow rate. The prediction of previous studies has not been observed and the expected nitrite accumulation did not take place in any point (Figure 6. 3). Another important factor when promoting AOB instead of NOB microorganisms has been the temperature. Thus, this parameter was increased from room temperature (20 ºC in periods I, II, III) to 30-31 ºC (periods IV, V, VI) because it was previously stated that a temperature around 30 ºC enhanced AOB microorganisms growth (Hellinga et al., 1998). Unfortunately, no rise in the nitrite concentration in the AOB reactor was detected and the effect of the temperature increase was not the previously reported.

In Figure 6. 1 the nitrogen cycle is depicted. With the previously reported operational conditions, a nitrite accumulation should be present (Reino et al., 2016). The lack of this expected compound indicated that the traditional 2-steps nitrification process might not have been followed.

Microbiological results

Cloning libraries

Microbiological tests were run to demonstrate the presence of the expected microorganisms. A PCR was carried out with a set of primers which encodes the gene *amoA.*

This gene is in charge of producing the enzime that catalyzes ammonium oxidation (Rotthauwe and Witzel, 1997). It is present in ß class of Proteobacteria, as well know examples, the genera of *Nitrosomonas* and *Nitrospira*. The employed set of primers was amoA-1 F (as forward primer) and amoA-2 R (as reverse primer) and matches with a specific fragment of the gene of 491 base pairs (bp). In Figure 6. 4 the photography of the agarose gel of the PCR products is depicted.
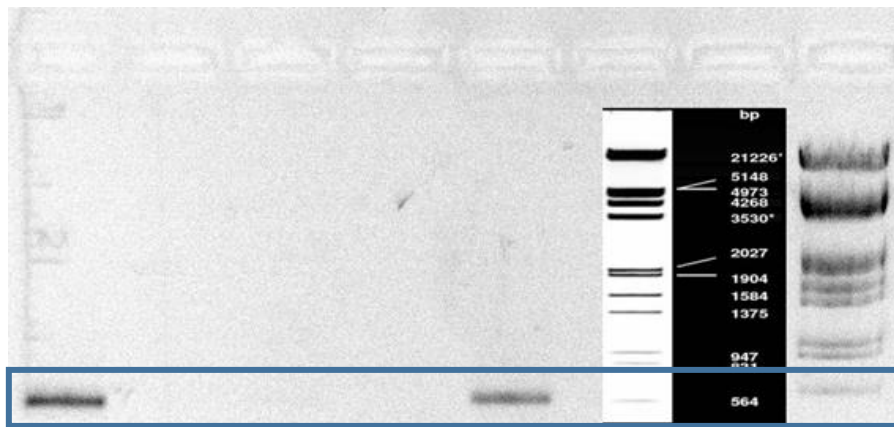
Figure 6. 4. Picture of the agarose gel where the PCR products with amoA-1 F and amoA-2 R were used as a set of primers applied to a sample collected from the A reactor.

The observed signal of the expected length of around 500 base pairs (bp) showed that in the sample existed DNA with the gene *amoA*. AOB microorganisms undoubtedly were present in Reactor A. PCR test did not provide relative/absolute concentration of the microorganisms in the active biomass of the reactor. For knowing further about the individuals present in the AOB reactor, a cloning library was elaborated by comparing the DNA strains of the obtained clones with those previously identified and collected in the public database, Basic Local Alignment Search Tool (BLAST). In Figure 6. 5 the result of this analysis is collected as a neighbor tree.
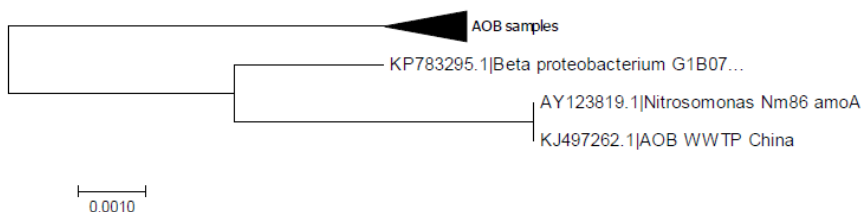
Figure 6. 5. Phylogenetic analysis of the amoA sequence family. The scale bar indicates the 0.1% of sequence divergence. Numbers in brackets indicated sequences per group. Amo: ammonium oxygenase.

The results collected in the neighbor tree of Figure 6. 5 indicated that all clones sequenced belonged to ß class of proteobacteria. The result of the comparison with other individuals present in BLAST database indicated that the individuals present in the A reactor were mostly related to the genera *Nitrosomonas* and *Nitrospira*.

Despite the absence of nitrite accumulation in any period of the experimental study, both analytical results and microbiological studies revealed that ammonium oxidizing populations were present in the Reactor A. Thus, a coexistence of any other nitrogen transformation mechanisms may be the explanation of the unexpected absence of nitrite accumulation. Very recently, the complete ammonium oxidation (comammox) has been demonstrated. This newly discovered microbiological process has been carried out by a bacterium belonging to *Nitrospira* genus. These microorganisms are capable of carrying out the two-steps ammonium oxidation into nitrite and then nitrate by an only single microorganism (Daims et al., 2015; van Kessel et al., 2015), instead of the different organisms involved as traditionally reported. Further studies, out of the scope of this work, confirmed via PCR the presence of this type of *Nitrospira* in the Reactor A in coexistence with the previously observed clones similar than *Nitrosomonas*.

FISH assays

FISH assays were carried out monthly during the continuous operation of the reactor. In Figure 6. 6, Figure 6. 7 and Figure 6. 8 are depicted the most representative pictures of those taken. In A column has been represented the whole active biomass found (green). B column overlaps the active biomass (green) and the signal related to the genus *Nitrosomonas* (red). It was only observed a low-intensity signal with the sample taken on day 48. In the previous cases, the signal of *Nitrospira* was negligible. C column overlaps the active biomass (green) and the signal related to the genus *Nitrospira* (blue). It was detected an abundant signal of this microorganism spread across the flocs in all three samples. The intensity of the signal increased with the operating time.
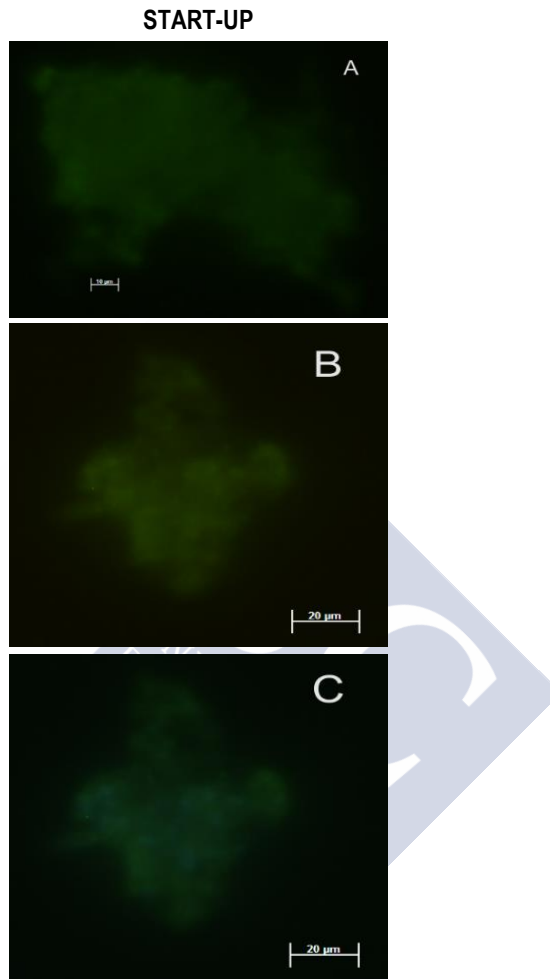
START-UP

Figure 6. 6 FISH images of the extracted biomass from the A reactor during the starting-up. A: Eubmix (green). B: Eubmix (green) + nso-1225 (red). C: Eubmix (green) + ntspa-0662 (blue).
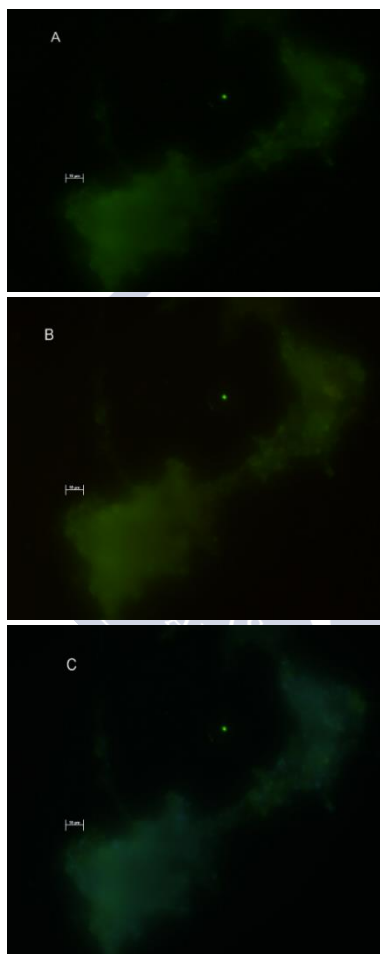
DAY 15

Figure 6. 7. FISH images of the extracted biomass from the A reactor during the day of operation 15th. A: Eubmix (green). B: Eubmix (green) + nso-1225 (red). C: Eubmix (green) + ntspa-0662 (blue).
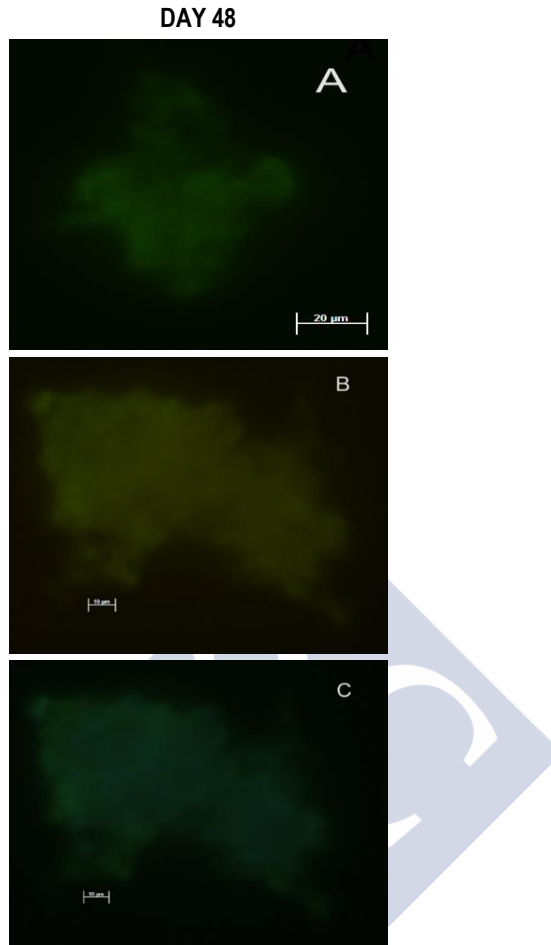
DAY 48

Figure 6. 8. FISH images of the extracted biomass from the A reactor during the day of operation 48th. A: Eubmix (green). B: Eubmix (green) + nso-1225 (red). C: Eubmix (green) + ntspa-0662 (blue).

Very recently, scientists discovered comammox, microorganisms able to completely transform ammonium into nitrate, bypassing nitrite. These microorganisms belonged to the genus *Nitrospira* (van Kessel et al., 2015). The results of the FISH assays showed a massive presence of microorganisms belonging to this genus in the samples. Analytical results were in accordance with these observations since nitrite was not detected in any point of the continuous operation of A the reactor. FISH analysis supported the hypothesis of the presence of comammox in the A reactor. The

presence of this newly discovered microorganism was confirmed via PCR in further experimental studies.

Reactor B

The HRT in the B reactor was maintained at 1 day, similarly than in the A reactor. The pH ranged into 7.2 and 8.3. DO was higher than in the case of the A reactor to enhance the growth of NOB microorganisms. Thus, detected DO values ranged into 35-55% of the saturation. The remaining ammonium from the A reactor has been fully oxidized with only traces detected in some of the samplings. Moreover, nitrite was hardly detected. In Figure 6. 9 the evolution trends of the nitrogen species in the NOB reactor are represented.
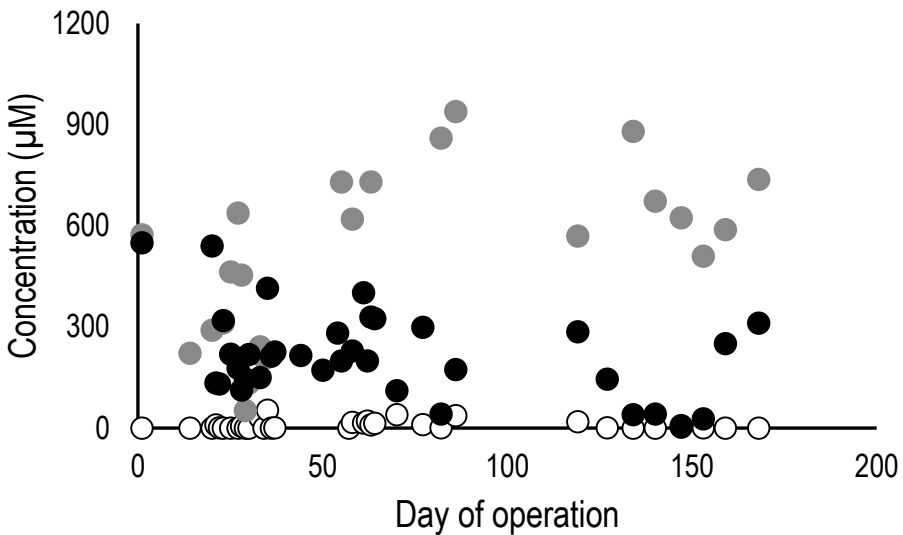
Figure 6. 9 Nitrogen species evolution in B reactor. (●) Ammonium in the influent; (○) ammonium in the effluent and (●) nitrate in the effluent

The absence of ammonium in the outlet of the B reactor indicated that the oxidation of the nitrogen species coming from the A reactor were fully achieved into nitrate. Moreover, it was detected a fraction of nitrogen elimination in the system since the

total nitrogen present in the influent of A reactor was higher than the detected in the effluent of the NOB of the B reactor.

Microbiological results

Similarly to A reactor, a PCR was carried out in the reactor B with a set of primers which encodes microorganisms like the included in the genus *Nitrospira* (Haaijer et al., 2013). These primers were 616F (as forward primer) and NTSPA712 (as reverse primer). Due to the low presence of biomass in this reactor, this PCR has to be carried out several times since the previous DNA extraction had not been conducted properly and no signal of PCR had been detected. In Figure 6. 10 is shown the photography of the agarose gel product of the successful PCR test.
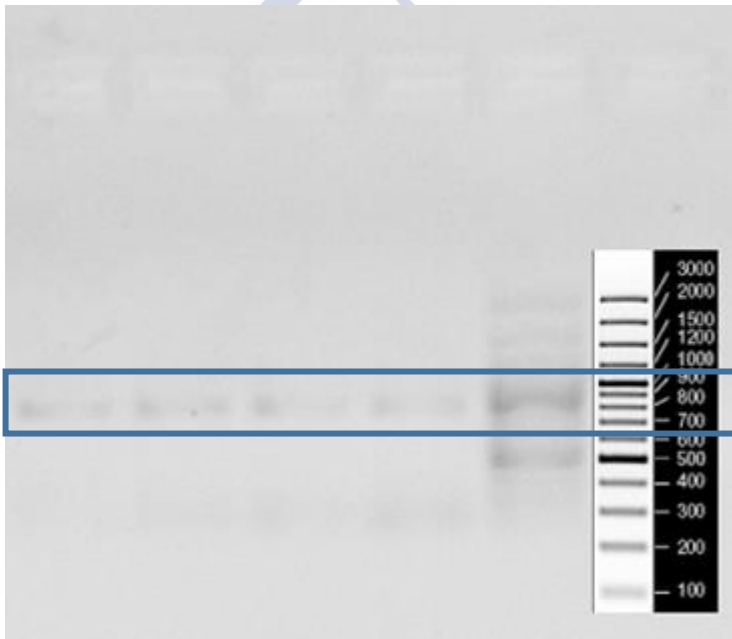

Figure 6. 10 Picture of the agarose gel where the PCR products with 616 F and NTSPA712 were used as a set of primers applied to a sample collected from the B reactor

The presence of microorganisms belonging to *Nitrospira* genus has been demonstrated via PCR since the obtained signal was observed in the expected 690 bp. The increase of nitrate in this NOB reactor supported the hypothesis of the

existence of these microorganisms. In contrast to AOB reactor, both analytical and microbiological behavior in the B reactor was according to the expected.

6.3.2. Discontinuous enrichment culture reactors

Due to the unexpected ammonium oxidation to nitrate in the continuous operation of the AOB reactor, an alternative enrichment technique had to be carried out.

Initially, it was planned to harvest pure cultures of *Methylomonas lenta* as representatives of MOB following a discontinuous growth strategy. Finally, it was decided to extend it to *Nitrosomonas europaea* as representatives of AOB due to the absence of nitrite observed when operating the continuous reactor. Figure 6. 11 includes pictures of the result of the growth of both AOB and MOB representatives. These microorganisms were later employed for performing further experiments.

6.3.1. *Nitrosomonas europaea*: minimum oxygen concentration requirements

Biological Oxygen Monitor (BOM) assays were performed to evaluate the minimum DO concentration needed to support biological oxidation of ammonium when *Nitrosomonas europaea* had to lead the oxidizing activity. The aim of this assay was to: I) demonstrate that oxygen availability was not a limitation in any of the subsequent experiments; II) find out the lower oxygen concentration required by *Nitrosomonas europaea* for carrying out an adequate activity. In Figure 6. 12 are summarized the experimental collected data.
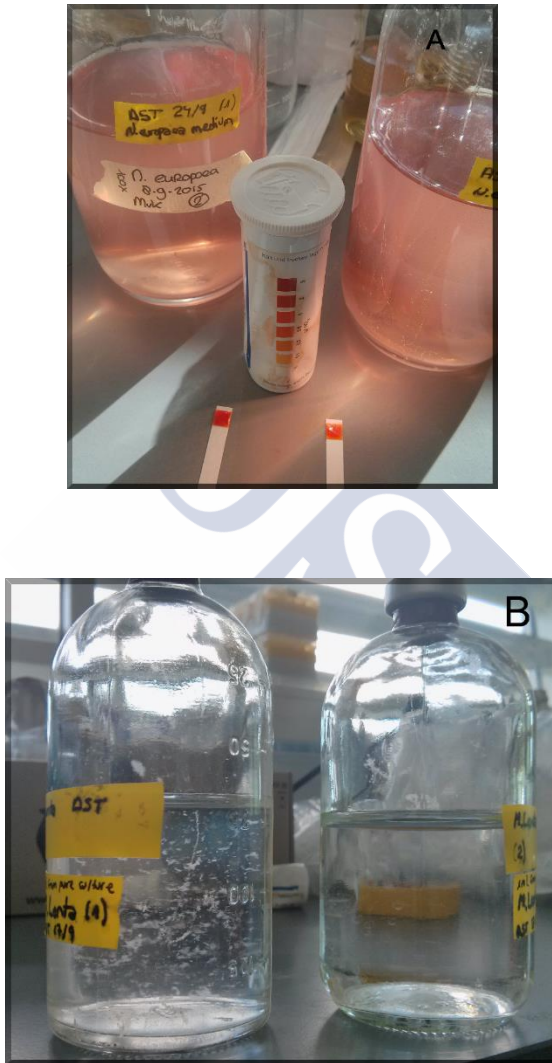
Figure 6. 11 A. Aspect of the culture of Nitrosomonas europaea when a proper activity was present as demonstrated by nitrite strips. Phenol red indicator provided the red color. B. Picture of the flocculent culture of Methylomonas lenta (on the left) showing flocculent biomass; contrasting with a failed harvest (on the right)
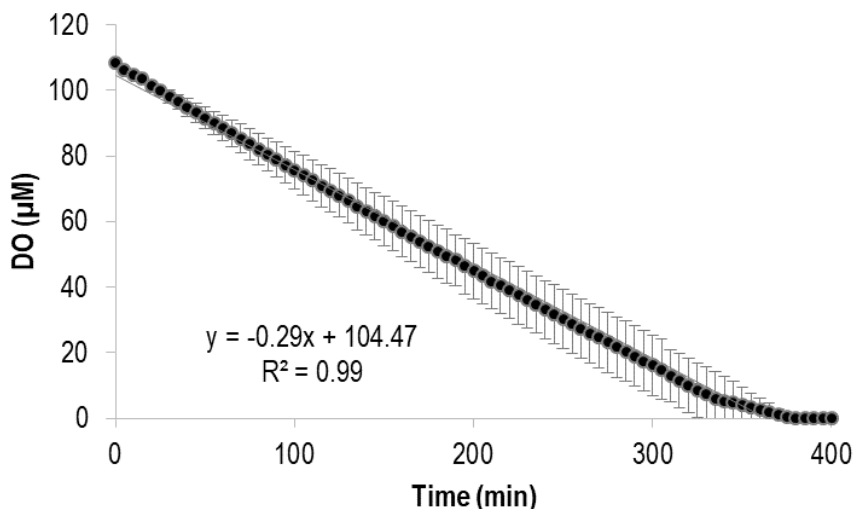
Figure 6. 12. Time evolution of dissolved oxygen (DO) in the liquid phase when a pure culture of *Nitrosomonas europaea* was present. The experiment was carried out in duplicate. Depicted are the average as dots and the standard deviation as error bars.

The experiment has been carried out in duplicate. Both experiments behaved in accordance with an averaged oxygen uptake rate (OUR) of 0.29 µM/min. DO was totally depleted accordingly to the accuracy of the employed probe. It indicated that the oxygen (from air) present in the headspace of the subsequent experiments will not be any limitation for the oxidation purposes. The obtained results were in accordance to Park et al., (2010) who detected a minimum DO concentration of 0.09 mg/L by using modelling tools instead of experimental data in a study involving a wider number of AOB.

6.3.2. *Nitrosomonas europaea*: methane plays a role as an inhibitor?

Batch experiments were carried out to assess whether methane plays a role in the ammonium oxidation when coexisting in the same ecosystem than *Nitrosomonas europaea*. Genes able to either ammonium oxidize (*amoA)* or methane oxidize (*pmoA*) have a similar structure and are evolutionarily related (Holmes et al., 1995). It was

reported that AOB have been able to oxidize methane and MOB were capable of oxidizing ammonium (Hyman and Wood, 1983).

Nitrosomonas europaea: ammonium oxidation as a function of methane concentration

It was studied the ammonium oxidation in four different levels of methane concentration in the headspace (0, 5, 10, 20 %CH$_4$) with ammonium concentration of 1000 µM NH$_4^+$ constant in all assays. It can be calculated the equivalent CH$_4$ concentration in the liquid phase, corresponding to 0, 0.8, 1.6, 3.2 mgCH$_4$/L, by means of Henry's law. In Figure 6. 13 are depicted the experimental results obtained.
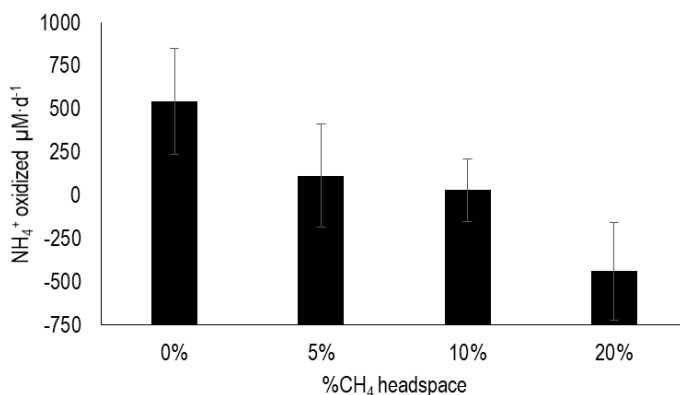

Figure 6. 13 Bar graph of ammonium oxidation by *Nitrosomonas europaea* as a function of methane concentration in the headspace (0-20%). All experiments were carried out in presence of 1000 µM NH$_4^+$ in the liquid phase.

As a general pattern, the lower methane concentration in the headspace the higher AOR was detected. It was observed an ammonium oxidation rate (AOR) of 544 µM/d when methane in the headspace was absent. This rule was fulfilled in all assays except for the highest methane concentration of 20% which showed a different behavior. It was obtained an AOR below zero (-437 µM/d). This value appeared as a consequence of the generation of ammonium in the medium. It was due to the poisoning process conducted in presence of a larger methane concentration than *Nitrosomonas*

europaea can handle. As a result of the microorganisms' death, ammonium was released in the medium coming from the cell debris.

The kinetics of the oxidizing process has been decelerated with the presence of only 5% of methane in the headspace. Thus, only 20% of the AOR in this latter case when compared with the study in total absence of methane. The existence of an environment of competitive inhibition (Suzuki et al., 1976) by methane was only real in the range of 0-5% of methane. Higher levels inhibited the microorganisms able to carry out this oxidation process. The activity was almost negligible when methane was initially present at a concentration of 10% onwards.

These results were in accordance with the limited literature available on this topic. Suzuki et al., (1976) studied the inhibition ability of methane, carbon dioxide and methanol. Similarly, than the current experimental results, methane showed a total inhibition of ammonium oxidation with *Nitrosomonas europaea*, especially at lower ammonium concentration values than 330 μM NH_4^+. In this current study, not only an inhibition was detected also a poisoning process has been observed. Methane acted as an inhibitor not only with *Nitrosomonas europaea* also other AOB species containing the *amoA* gene such as *Nitrosococcus oceanus* behaved in a similar way when this compound was in the medium (Ward, 1987).

Nitrosomonas europaea: methane as an alternative substrate to ammonium

Hyman and Wood, (1983) stated that methane could be oxidized by the gene *amoA* present in *Nitrosomonas europaea*. The presence of both substrates ammonium and methane created an environment of ammonium inhibition by methane (Suzuki et al., 1976). Methane evolution was analyzed for assessing the less favorable substrate for those available in the medium. Figure 6. 14 showed the methane oxidation activity as a function of the methane availability in the headspace, ammonium was initially constant at 1000 μM NH_4^+ in all bottles.
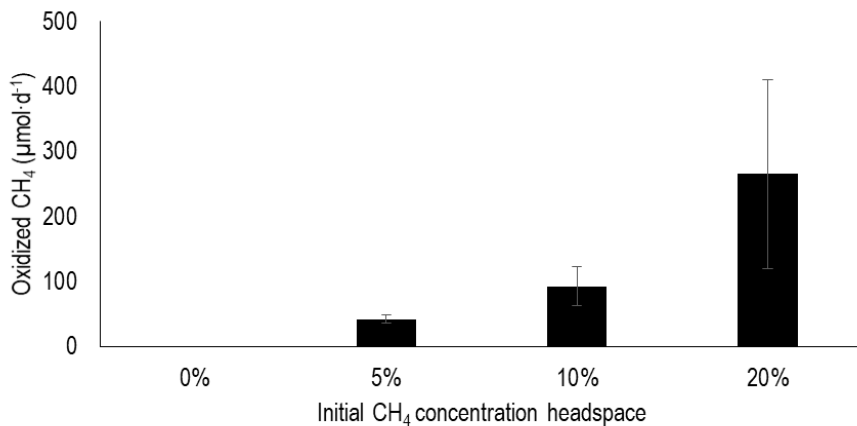
Figure 6. 14. Methane oxidation by *Nitrosomonas europaea* as a function of methane concentration in the headspace (0-20%). All experiments were carried out in presence of 1000 μM NH$_4^+$ in the liquid phase.

The obtained results indicated that the higher initial methane availability in the gas phase the largest oxidation rate when the initial methane concentration ranged into 0-20%. It confirmed that methane can be a suitable substrate for *Nitrosomonas europaea* as previously stated (Hyman and Wood, 1983; Stein et al., 2012). Moreover, when the lowest methane availability was present *Nitrosomonas europaea* showed a clear preference for ammonium over methane, as expected due to the *amoA* gene action. In the first three methane concentration levels (0, 5 and 10%) the trends were similar, behaving as a linear curve. Nevertheless, when the initial methane availability in the headspace was of 20% a different pattern was observed (Figure 6. 15).
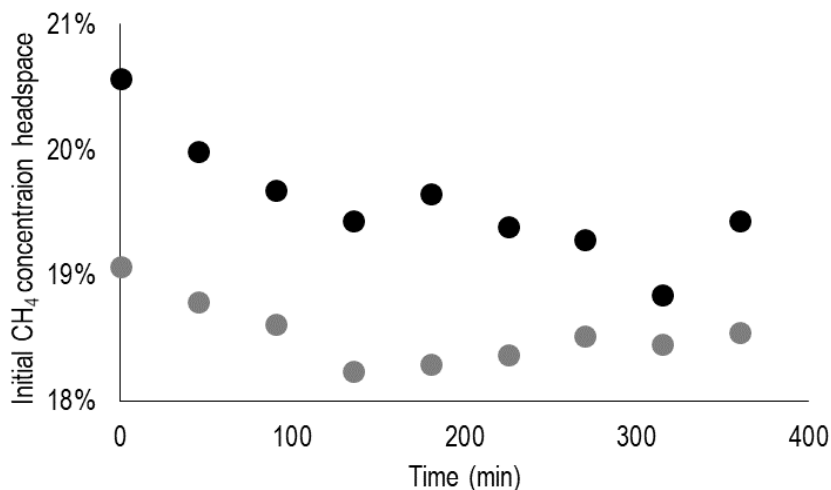
Figure 6. 15. Time evolution of methane in the headspace when its concentration was of 20% at the beginning of the experiment. A pure culture of *Nitrosomonas europaea* was present in liquid phase. The experiment was carried out in duplicate. Duplicate 1 (black); duplicate 2 (grey).

The largest extension of these oxidation activities were carried out only for the first 135 minutes of experiment. Later, the methane oxidation activity either stopped or dramatically decreased. In Figure 6. 15 is depicted the most representative example of this behavior when the initial methane concentration in the headspace was of 20%. Such a decrease in both duplicates of the experiment confirmed a stage of poisoning of the biomass as abovementioned.

6.3.3. *Methylomonas lenta*: minimum oxygen concentration requirements

BOM assays were performed to evaluate the minimum DO concentration needed to support a biological oxidation of methane by *Methylomonas lenta*. The aim of this assay was to: I) demonstrate that oxygen availability was not a limitation in any of the subsequent experiments; II) find out the lower oxygen concentration required by *Methylomonas lenta* for carrying out an adequate activity. In Figure 6. 16 are shown the graphical curves extracted.
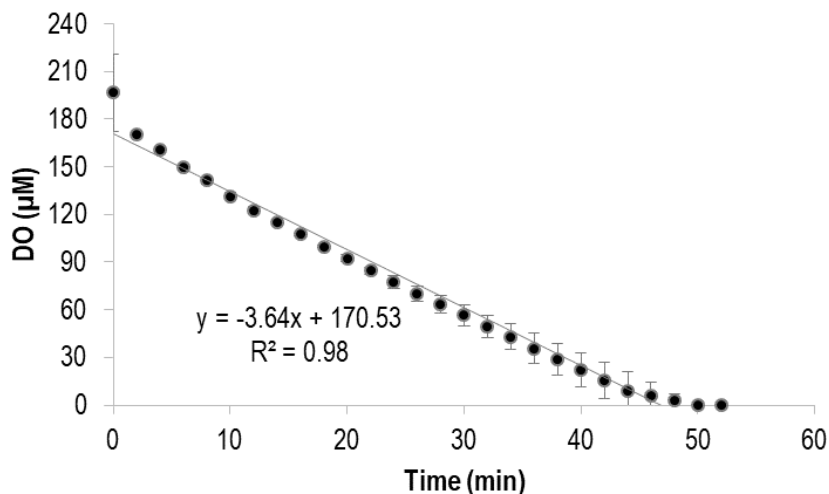
Figure 6. 16 Time evolution of dissolved oxygen (DO) in the liquid phase when a pure culture of Methylomonas lenta was present. The experiment was carried out in duplicate. Depicted are the average as dots and the standard deviation as error bars.

The experiment has been carried out in duplicate. Both experiments behaved in accordance with an oxygen uptake rate (OUR) of 3.64 µM/min. In the case of *M. Lenta*, DO was totally depleted ten orders of magnitude faster than *N. europaea*, in the experimented conditions. It indicated that the oxygen present in the headspace of the subsequent experiments will not be any limitation for the oxidation purposes. The obtained results were according to Hernandez et al., (2015) who published results of MOB for DO evolution with a total exhaustion of this compound.

6.3.4.   *Methylomonas lenta:* methane oxidizing activity inhibition by ammonium

Batch experiments were conducted to evaluate the role of ammonium and nitrite (oxidation product of ammonium) when coexisting with methane in the same ecosystem than *Methylomonas lenta*.

Methylomonas lenta: methane oxidation as a function of ammonium concentration

Methane oxidation activity was studied as a function of ammonium concentration in liquid phase. In Figure 6. 17 experimental values are depicted.
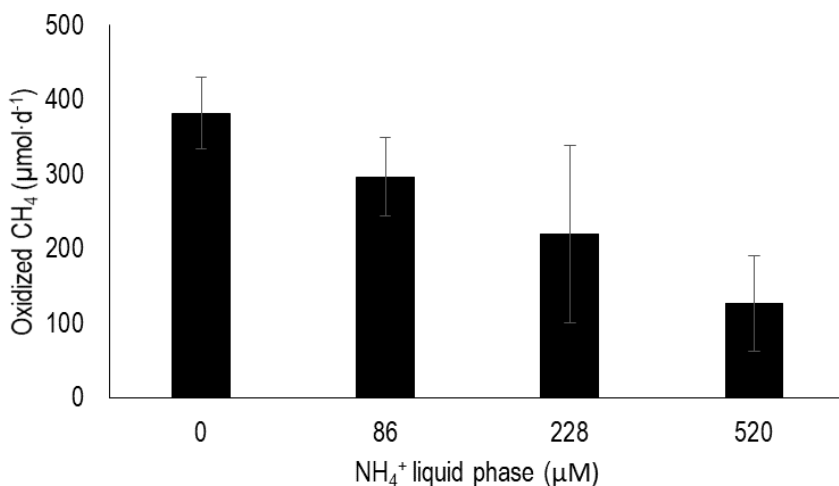

Figure 6. 17 Methane oxidation as a function of the ammonium (0-520 µM) in the liquid phase. Each batch assay was performed in triplicate. The initial methane concentration in all assays was of 1% in the headspace.

A maximum methane oxidation rate (MOR) of 0.38 mmol/d was achieved with a total absence of ammonium in the liquid phase was set up. Later, the higher ammonium concentration the lower capacity of oxidizing methane. The maximum ammonium concentration values of those tried (520 µM NH_4^+) corresponded to the minimum methane-oxidizing rate of 0.13 mmol/d, equivalent to an activity of 33% when compared to the rate in absence of ammonium. The coexistence of two oxygen demanding substrates as long as the ability of the gene *pmoA* (present in methanotrophs such as *Methylomonas lenta*) for oxidizing methane and ammonium (Holmes et al., 1995) explained the observed results. As previously reported, *pmoA* gene has been able to oxidize ammonium, but at least two orders of magnitude slower than in the case of methane as substrate (Hanson and Hanson, 1996). Thus, a noticeably decreased methane oxidation was detected in presence of both ammonium

and methane when concentrations of 520 µM NH_4^+ or lower were present. In this case, it was not detected any poisoning episode in contrast to the observed results for the previous experiments when *Nitrosomonas europaea* handled with 20% of methane in the headspace.

King and Schnell, (1994) carried out similar batch experiments with *Methylobacter albus BG8* and *Methylosinus trichosporium OB3b* instead of *M. lenta*. It was detected a similar pattern in both cases. Ammonium acted as an inhibitor in accordance with the results obtained in the current experiment. Nevertheless, in the case of *M. albus* the inhibitory effect was much sharper than in the case of *M. trichosporium*. As a general rule, ammonium behaved as an inhibitory substance for the gene *pmoA*. The magnitude of the decrease in the oxidation activity strongly depended on each specie.

Methlylomonas lenta: ammonium as an alternative substrate to methane
Apart from methane, it was evaluated the trends of the other substrate present in the medium, ammonium. The tested concentrations were 0, 86, 229 and 520 µM NH_4^+. In Figure 6. 18 are shown the main results.
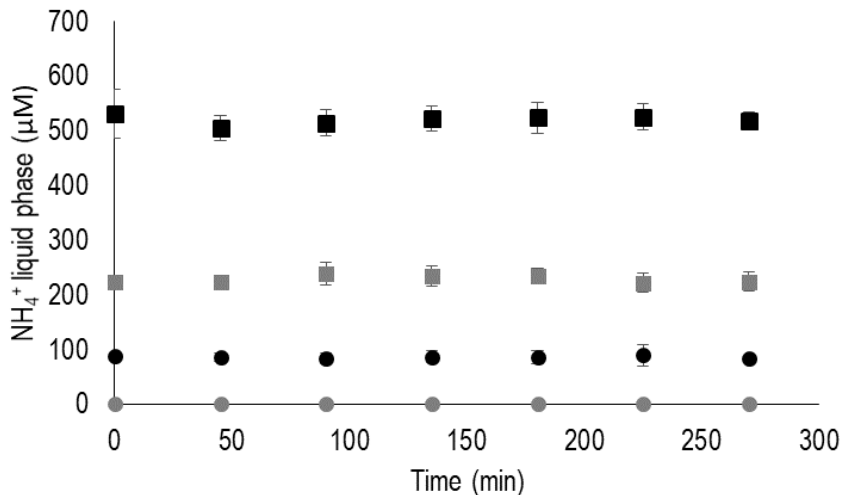

Figure 6. 18. Batch assays of Methylomonas lenta oxidation activity when ammonium acted as a substrate. Each assay has been performed in triplicate. It was depicted the averaged time evolution of the ammonium concentration. The initial ammonium concentration in each experiment was of 0 (●), 86 (●), 229 (■) and 520 (■) µM NH_4^+.

It was not detected any ammonium variation for any of the three different experimented levels of ammonium in the liquid phase. Neither ammonium oxidation nor poisoning of the methane-oxidizing biomass have been observed. Thus, the experimented the capacity of the gene *pmoA* (present in *Methylomonas lenta*) of oxidizing ammonium (Hanson and Hanson, 1996) was not detected with the very accurate analytical method employed for both methane and ammonium. For this reason, *Methylomonas lenta* showed a clear preference for methane as substrate instead of ammonium when both compounds were present.

Methlylomonas lenta: nitrite plays a role?

Nitrite has been traditionally presented as the product of AOB microorganisms when oxidizing ammonium. For this reason, the presence of this species in a medium where MOB and AOB microorganisms are present is expectable. A set of batch experiments were performed to assess if this species might play any role either as an enhancer or inhibitor or without any role. Moreover, in some recipes for its culture nitrite has been advised to be present in the medium (Hoefman et al., 2014).
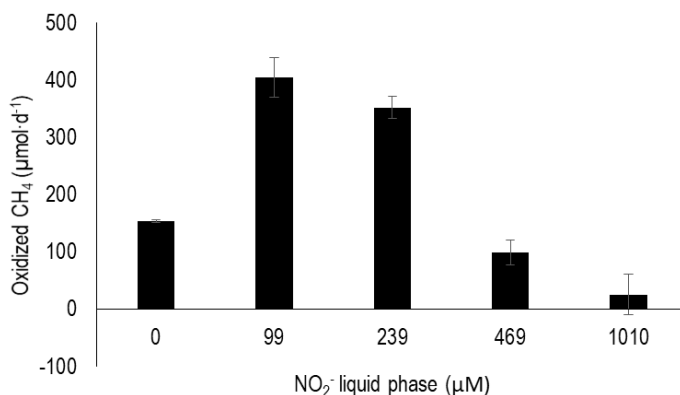
Figure 6. 19 Methane oxidation by Methylomonas lenta as a function of the nitrite concentration present in the liquid medium (0-1010 μM CH₄). Each experiment has been conducted in duplicate. All experiments were carried out in presence of the 1%CH₄ in the headspace.

The detected behavior will be divided into three stages for easily explaining reasons. I) When nitrite was absent in the medium a MOR of 0.15 mmol/d was detected and it

was considered the comparative basis for other observed activities. II) When 99 and 239 μM NO$_2^-$ were present a MOR enhancement of 262% and 228%, respectively has been observed. III) From that nitrite value onwards, a MOR inhibition was detected. When the established nitrite value was of 1010 μM NO$_2^-$ the MOR was almost negligible. The obtained results indicated that at nitrite concentrations no longer than 239 μM played as an enhancer for the oxidation activity of *Methylomonas lenta*.

King and Schnell, (1994) studied the methane oxidation with other types of methanotrophs such as *Methylobacter albus BG8* and *Methylosinus thrichosporium OB3b* at nitrite concentrations ranging into 0 and 1000 μM NO$_2^-$. In the case of *M. albus* the inhibition was observed at lower nitrite concentrations than *M. lenta*. Nevertheless, *M. trichosporium* showed a much lower inhibition rate than both *M. albus* and *M. lenta*. It indicated that the effect of nitrite in the methane oxidation strongly depended on the involved specie. On the other hand, methane oxidation in a humisol of Canada was investigated. In this case, an inhibitory effect of nitrite was not observed when nitrite was lower than 260 μM NO$_2^-$ (Dunfield and Knowles, 1995). Hoefman et al., (2014) previously found *Methylomonas lenta* in denitrification tanks of WWTP. In this latter facilities, nitrite has been typically formed during the denitrification process in such a range included in the previously described "enhancing stage" (Figure 6. 19). Methane might be present due to the absence of oxygen and it has been present even in the raw sewage (Daelman et al., 2012). Hence, the conditions present in the denitrification tanks seemed to be suitable for a larger methane oxidation by *M.lenta* than in other locations of a WWTP where nitrite has not been typically present.

6.4. Conclusions

Ammonium oxidation was not stopped in nitrite during the continuous enrichment operation. Both FISH analysis and cloning libraries confirmed the presence of representatives of ammonium oxidizers. Nevertheless, FISH results suggested an increasing relative presence of microorganisms included in the genus *Nitrospira*. In further studies, out of the scope of this study, it was confirmed via PCR the presence a newly discovered microorganism, included in the genus *Nitrospira*, able to perform the two steps involved in ammonium oxidization by one single microorganism step

(comammox).

Both ammonium and methane oxidation cultures have been obtained by growing discontinuously pure cultures of *Nitrosomonas europaea* and *Methylomonas lenta*, respectively.

The coexistence of 1000 μM of ammonium (liquid phase) and methane ranged into 0-20% (headspace) as substrates in presence of *Nitrosomonas europaea* generated an environment of competitive inhibition when 0-5% of methane was present. From that concentration of methane onwards, the ammonium oxidizing activity was either stopped or even a massive biomass poisoning has been detected at 20% of methane.

Ammonium ranged into 0-520 μM NH_4^+ (liquid phase) and 1% of CH_4 (headspace) in the simultaneous presence of *Methylomonas lenta* as a pure culture resulted in: the higher ammonium concentration the lower methane oxidation activity. It indicates the inhibitory effect of ammonium for *Methylomonas lenta.*

Nitrite, as a product of ammonium oxidation, in the range of 0-1020 μM NO_2^- (liquid phase) and 1% of CH_4 (headspace) in concurrence of *Methylomonas lenta* behaved as follows: up to 239 μM acted as an enhancer of ammonium oxidation. From that concentration onwards, nitrite acted as an inhibitor of ammonium oxidation.

BOM assays for both ammonium and methane oxidizers elucidated that it was not required a minimum DO concentration for carrying out the metabolic activity of both AOB and MOB. Oxygen was not a limiting compound in any of the performed experiments.

GENERAL CONCLUSIONS

The main conclusions of this PhD thesis, entirely focused on the use of different Membrane Bioreactor (MBR) systems and processes, in which either hybrid systems combining biofilms and suspended biomass; anaerobic methanogenic roughing stages; or the growth of dedicated microorganisms was carried out. The main conclusions of the four experimental chapters are detailed below:

CHAPTER 3. Municipal wastewater treated by a hybrid membrane bioreactor as a compact system

A newly developed hybrid-MBR was proposed as a compact system for treating low strength primary treated wastewater. The use of this system, combining flocculent and attached biomass in biofilms, could be a good option for increasing either the hydraulic capacity or the organic removal rate of the Activated Sludge system used at that time. This hybrid system could be used in medium and large-scale urban WWTPs.

Full solids retention and ammonium nitrification were attained in the reactor. Nonetheless, a bottleneck was detected when studying nitrogen denitrification. A combined effect of the low hydraulic capacity of the internal recycling pump, a low COD/TN ratio and a scarce alkalinity, typical of the wastewater of this area (NW Spain), led to low denitrification efficiencies, and must be considered when nitrogen removal capacity of the biological stage should be considered.

Even considering the low operating pH observed during different periods of the experiment, membrane performance showed remarkable efficiency indicators: high permeability and a low fouling rate.

CHAPTER 4. Assessment of a combined UASB and MBR process treating wastewater from a seafood industry at different temperatures

The use of a combined two stages UASB and MBR system was proposed for treating an industrial wastewater stream generated in a seafood processing factory. The studied two-stages UASB and MBR was robust and reliable, and it has been capable

of removing 94±4% of the incoming COD and the 100% of TSS present in the wastewater within the whole experimental period. The combined system was capable of counteracting the COD overloads to the MBR polishing system, when the UASB efficiency diminished. These values always fulfilled the discharge limits of the factory where the system was located.

The presence of biocides in the industrial wastewater stream, in a concentration probably larger than 30 mg/L, inhibited both anaerobic treatment and nitrification process. Methanogenic phase was recovered in the anaerobic treatment after 50 days and an irreversible nitrification drop in the post-treatment was experimented.

Temperature control and its stability over time have been essential parameters for achieving the maximum organic removal rate, 6 kgCOD/(m^3·d) at 30 ºC, corresponding to more than the 90% of the fed Organic Loading Rate (OLR) in the UASB system. Observed methanation percentages were larger than 90%. In these conditions, larger OLR could be applied, since the OLR fed to the UASB was limited by the incoming wastewater COD concentration and the filtration capacity of the membrane used during the experiments. On the other hand, biomass yield, around 0.18 gVSS/gCOD, was lower than that currently associated to aerobic MBR systems. The removal of a large fraction of the incoming COD in the UASB, made feasible to diminish sludge production.

CHAPTER 5. MBR as post-treatment of anaerobically treated effluents

Sewage treated anaerobically at low temperature contains dissolved methane, which should be removed in order to reduce greenhouse gas emissions (GHG) of this type of wastewater treatment broadly used in different temperate/warm climate countries. In this research, a Membrane Bioreactor (MBR) post-treatment was proposed that is able to simultaneously remove methane and nitrogen by implementing biological processes involving methane oxidation coupled to denitrification. The pre-anoxic MBR post-treatment was capable of consistently removing 80% of dissolved methane, with peaks up to 95% at 17-23 °C. Moreover, biological treatment partially removed nitrogen, up to 15-20 mg TN·L^{-1}, by coupling methane oxidation and denitrification. Recycle ratio and dissolved oxygen played a crucial role to govern the denitrification mechanisms. Mass balances indicated that the fate of a fraction of the eliminated

methane was still unknown; therefore, further studies are required to reveal its destination.

Microbiological studies elucidated that AMO-D was present due to the great abundance of the most representative microorganisms. On the other hand, batch assays and microbiological tests undoubtedly demonstrated the presence of DAMO activity. This study opened the door to reducing the GHG impacts associated to the anaerobic treatment of sewage in temperate and warm climates countries. The elimination of the majority of the dissolved methane, suspended solids and the remaining biodegradable COD of the anaerobically treated effluents, converted this treatment as friendly from the ecological point of view, reducing part of the nitrogen contained in sewage.

CHAPTER 6. Overview of the competition among oxidizers of methane and ammonium in an oxygen scarcity environment

Filtration membranes allows the selective retention of compounds, cells can be retained since their size is typically smaller than the membrane porous size. This fact was view as an opportunity when culturing and harvesting slow growing microorganisms.

A battery of two complete stirred tank reactors were used for promoting the growth of either ammonium or nitrite oxidizing bacteria, respectively in. A mixed culture of a previous harvested in a previous research has been employed as inoculum. Operational conditions, such as temperature or solids retention time, aiming at obtaining those above-described harvests were set. Nevertheless, a quick growth of commamox representatives was observed. Complete Ammonium Oxidation (commamox) are newly discovered microbes belonging to the genus *Nitrospira* able to oxidize ammonium into nitrate themselves. Thus, the subsequent steps of ammonium and nitrite oxidation could proceed in one single microorganism. Although the commamox culture was accidental in the studied system, the capacity of membranes of enriching those microorganisms of interest has been proven.

CONCLUSIONES GENERALES

Las principales conclusiones de esta tesis doctoral, totalmente focalizada en el uso de diferentes sistemas y procesos basados en Biorreactores de Membrana (BRM), se detallan en los siguientes párrafos. Durante los trabajos experimentales, se han operado sistemas híbridos que combinan biopelículas y biomasa suspendida; otros cuya finalidad ha sido la adecuación de aguas residuales tratadas anaeróbicamente; y también se han cultivado microorganismos específicos. Las principales conclusiones de los cuatro capítulos experimentales se detallan a continuación:

CAPÍTULO 3. Aguas residuales municipales tratadas por un biorreactor compacto híbrido de membranas.

Se ha propuesto un BRM compacto híbrido recientemente desarrollado para la depuración de aguas residuales tratadas mediante decantación primaria. El uso de este sistema, combinando biomasa floculenta y adherida en biopelículas, se había planteado como una buena alternativa para aumentar tanto la capacidad hidráulica como la tasa de eliminación de contaminantes orgánicos con respecto al sistema de lodos activados, utilizado en ese momento. Este sistema híbrido podría usarse en EDAR urbanas de media y gran escala.

La retención total de sólidos y la nitrificación total de amonio se ha alcanzado durante toda la etapa experimental. No obstante, la desnitrificación del nitrógeno ha resultado ser un cuello de botella en el sistema. El efecto combinado de la baja capacidad de bombeo de la bomba de recirculación interna, una baja relación DQO/NT y una alcalinidad escasa, típica de las aguas residuales de esta área (NO de España), condujo a bajas eficiencias de desnitrificación. Este efecto debe considerarse cuando la eliminación de nitrógeno sea uno de los límites de vertido a cumplir.

Incluso teniendo en cuenta el bajo pH de funcionamiento observado durante los diferentes períodos de la fase experimental, el rendimiento de la membrana mostró notables indicadores de eficiencia: alta permeabilidad y baja tasa de ensuciamiento.

CAPÍTULO 4. Evaluación de un proceso combinado de UASB y BRM que trata aguas residuales de una industria de procesado de productos del mar a diferentes temperaturas.

El uso de un sistema combinado de dos etapas, UASB y BRM, fue propuesto para tratar una corriente de aguas residuales industriales generada en una fábrica de procesamiento de productos del mar. El reactor de dos etapas UASB y BRM ha resultado ser robusto y fiable, siendo sido capaz de eliminar 94 ± 4% de la DQO entrante y el 100% de SST presente en las aguas residuales en todo el período experimental. El sistema combinado ha contrarrestado las sobrecargas de DQO en el reactor de adecuación del efluente BRM, cuando la eficiencia de UASB disminuyó. El efluente tratado siempre cumplió los límites de descarga de la fábrica donde se instaló el sistema.

La presencia de biocidas en la corriente de aguas residuales industriales, en una concentración probablemente mayor a 30 mg/L, inhibió tanto el tratamiento anaeróbico como el proceso de nitrificación. La fase metanogénica se recuperó en el tratamiento anaeróbico después de 50 días. Por el contrario, se experimentó una caída de la nitrificación irreversible en el post-tratamiento.

El control de temperatura y su estabilidad a lo largo del tiempo han sido parámetros esenciales para alcanzar la máxima tasa de eliminación orgánica, 6 kgDQO/(m^3·d) a 30 ºC, que corresponde a más del 90% de la Velocidad de Carga Orgánica (VCO) en el sistema UASB. Los porcentajes de metanización observados fueron mayores al 90%. En estas condiciones, se habría podido aplicar una VCO más grande, ya que la VCO alimentada al UASB estaba limitada por la concentración de DQO del agua residual entrante, y por la capacidad de filtración de la membrana utilizada. Por otro lado, el rendimiento de biomasa, alrededor de 0.18gSSV/gDQO, fue menor que el actualmente asociado a los sistemas BRM aeróbicos. La eliminación de una gran fracción de la DQO entrante en el UASB hizo posible disminuir la producción de lodo.

CAPÍTULO 5. BRM como tratamiento posterior de efluentes tratados anaeróbicamente.

Las aguas residuales tratadas anaeróbicamente a baja temperatura contienen metano disuelto, que debe eliminarse para reducir las emisiones de gases de efecto

invernadero (GEI) de este tipo de tratamiento de aguas residuales, tan ampliamente utilizado en diferentes países de clima templado/cálido. En esta investigación, se propuso un post-tratamiento BRM que es capaz de eliminar simultáneamente metano y nitrógeno mediante la implementación de procesos biológicos que implican la oxidación de metano junto con la desnitrificación (AMO-D). El post-tratamiento, con una configuración pre-anóxica del BRM, fue capaz de eliminar consistentemente el 80% del metano disuelto, con picos de hasta el 95% a 17-23 °C. Además, el tratamiento biológico eliminó parcialmente el nitrógeno, hasta 15-20 mg de NT/L, mediante el acoplamiento de la oxidación del metano y su empleo en la desnitrificación como dador de electrones. La relación de recirculación y el oxígeno disuelto jugaron un papel crucial en el reparto de importancia de los mecanismos de desnitrificación implicados. Los balances de masa indicaron que el destino de una fracción del metano eliminado todavía era desconocido; por lo tanto, se requieren más estudios para revelar su destino.

Los estudios microbiológicos elucidaron que AMO-D estaba indudablemente presente debido a la gran abundancia de sus microorganismos más representativos. Por otro lado, ensayos en discontinuo y pruebas microbiológicas demostraron la presencia de actividad anaerobia de desnitrificación con metano, DAMO. Este estudio abrió la puerta a la reducción de los impactos de GEI asociados al tratamiento anaeróbico de las aguas residuales en los países con climas templados y cálidos. La eliminación de la mayoría del metano disuelto, los sólidos suspendidos y de la DQO biodegradable remanente de los efluentes tratados anaeróbicamente, convirtieron este tratamiento en amigable desde el punto de vista ecológico, reduciendo parte del nitrógeno contenido en las aguas residuales.

CAPÍTULO 6. Descripción general de la competencia entre los oxidantes de metano y amonio en un entorno de escasez de oxígeno.

Las membranas de filtración permiten la retención selectiva de ciertos compuestos por el tamaño de partícula. Las células pueden retenerse ya que su tamaño es típicamente más pequeño que el tamaño poroso de la membrana. Este hecho fue visto como una oportunidad para cultivar y cosechar microorganismos especializados de crecimiento lento.

Se utilizaron dos reactores de mezcla completa acoplados en serie, para promover el crecimiento de bacterias oxidantes tanto de amonio como de nitrito, respectivamente. Se utilizó como inóculo un cultivo mixto de un lodo procedente de una investigación anterior. Se establecieron las condiciones operativas, tales como la temperatura o el tiempo de retención de sólidos, con el objetivo de obtener los cultivos específicos objetivo del estudio. Sin embargo, se observó un rápido crecimiento de representantes de commamox. Los que ejecutan la oxidación completa de amonio (commamox) son microorganismos recientemente descubiertos que pertenecen al género *Nitrospira* y que pueden oxidar el amonio hasta nitrato con solo un microorganismo. Aunque el cultivo de commamox fue accidental en el sistema estudiado, se ha demostrado la capacidad de las membranas de enriquecer el lodo en esos microorganismos.

CONCLUSIÓNS XERAIS

As principais conclusións desta tese de doutoramento, totalmente enfocada no uso de diferentes sistemas e procesos baseados en Biorreactores de Membrana (BRM), detállanse nos seguintes párrafos. Durante os traballos experimentais, operáronse sistemas híbridos que combinaban biopelículas e biomasa suspendida; outros cuxa finalidade foi a adecuación de augas residuais tratadas anaeróbicamente; e tamén cultiváronse microorganismos específicos. As principais conclusións dos catro capítulos experimentais detállanse de aquí en adiante:

CAPÍTULO 3. Augas residuais municipais tratadas por un biorreactor compacto híbrido de membranas.

Propúxose un BRM compacto híbrido recentemente desenvolvido para a depuración de augas residuais tratadas mediante decantación primaria. O uso deste sistema, combinando biomasa floculenta e adherida en biofilmes, presentouse como unha boa alternativa para aumentar tanto a capacidade hidráulica como a taxa de eliminación de contaminantes orgánicos, con respecto ao sistema de lodos activados, empregado nese momento. Este sistema híbrido podería empregarse en EDAR urbanas de media e gran escala.

A retención total de sólidos e a nitrificación total do amonio acadouse durante toda a etapa experimental. Entrementres, a desnitrificación do nitróxeno foi unha limitación no sistema. O efecto combinado da baixa capacidade de bombeo da bomba de recirculación interna, unha baixa relación DQO/NT e unha alcalinidade escasa, típica das augas residuais desta área (NO de España), conduciu a baixas eficiencias na desnitrificación. Este efecto debe considerarse cando a eliminación de nitróxeno sexa un dos límites de vertido a cumprir.

Incluso tendo en conta o baixo pH de funcionamento observado durante os diferentes períodos da fase experimental, o rendemento da membrana mostrou notables indicadores de eficiencia: alta permeabilidade e baixa taxa de ensuzamento.

CAPÍTULO 4. Avaliación dun proceso combinado de UASB e BRM que trata augas residuais dunha industria de procesado de produtos do mar a diferentes temperaturas.

O uso dun sistema combinado de dúas etapas, UASB e BRM, foi proposto para tratar unha corrente de augas residuais industriais xerada nunha fábrica de procesamento de produtos do mar. O reactor de dúas etapas UASB e BRM foi robusto e fiable, sendo sido capaz de eliminar 94 ± 4% da DQO entrante e o 100% dos SST presente nas augas residuais en todo o período experimental. O sistema combinado contrarrestou as sobrecargas de DQO no reactor de adecuación do efluente BRM, cando a eficiencia de UASB diminuíu. O efluente tratado sempre cumpriu os límites de descarga fixados para a fábrica onde se instalou o sistema.

A presenza de biocidas na corrente de augas residuais industriais, nunha concentración probablemente maior a 30 mg/L, inhibiu tanto o tratamento anaeróbico como o proceso de nitrificación. A fase metanoxénica recuperouse no tratamento anaeróbico logo de 50 días. Pola contra, experimentouse unha caída da nitrificación irreversible no pos-tratamento.

O control de temperatura e a súa estabilidade ao longo do tempo foron parámetros esenciais para acadar a máxima taxa de eliminación de contaminación orgánica, 6 kgDQO/(m^3·d) a 30 ºC, que corresponde a máis do 90% da Velocidade de Carga Orgánica (VCO) no sistema UASB. As porcentaxes de metanización observadas foron maiores ao 90%. Nestas condicións, púidose ter aplicado unha VCO máis grande, xa que a VCO alimentada ao UASB estaba limitada pola concentración de DQO da auga residual entrante, e pola capacidade de filtración da membrana utilizada. Por outra banda, o rendemento de biomasa, arredor de 0.18gSSV/gDQO, foi menor que o actualmente asociado aos sistemas BRM aeróbicos. A eliminación dunha gran fracción da DQO entrante no UASB fixo posible diminuír a produción de lodo.

CAPÍTULO 5. BRM como tratamento posterior de efluentes tratados anaeróbicamente.

As augas residuais tratadas anaeróbicamente a baixa temperatura conteñen metano disolto, que debe eliminarse para reducir as emisións de gases de efecto invernadoiro (GEI) deste tipo de tratamento de augas residuais, tan amplamente utilizado en

diferentes países de clima temperado/cálido. Nesta investigación, propúxose un pos-tratamento BRM que é capaz de eliminar simultaneamente metano e nitróxeno mediante a implementación de procesos biolóxicos que implican a oxidación de metano xunto coa desnitrificación (AMO-D). O pos-tratamento, cunha configuración pre-anóxica do BRM, foi capaz de eliminar ao longo do tempo o 80% do metano disolto, con picos de ata o 95% a 17-23 °C. Ademais, o tratamento biolóxico eliminou parcialmente o nitróxeno, ata 15-20 mg de NT/L, mediante o acoplamento da oxidación do metano e o seu emprego na desnitrificación como doador de electróns. A relación de recirculación e o osíxeno disolto xogaron un papel crucial no reparto da importancia dos mecanismos de desnitrificación implicados. Os balances de masa indicaron que o destino dunha fracción do metano eliminado aínda foi descoñecido; polo tanto, requírense máis estudos para revelar o seu destino.

Estudos microbiolóxicos revelaron que AMO-D estaba indubidablemente presente debido á gran abundancia das súas especies máis representativas. Doutra banda, ensaios en discontinuo e probas microbiolóxicas demostraron a presenza de actividade anaerobia de desnitrificación con metano, DAMO. Este estudo abriu a porta á redución dos impactos de GEI asociados ao tratamento anaeróbico das augas residuais nos países con climas temperados e cálidos. A eliminación da maioría do metano disolto, os sólidos suspendidos e da DQO biodegradable remanente dos efluentes tratados anaeróbicamente, converteron este tratamento en amigable desde o punto de vista ecolóxico, reducindo parte do nitróxeno contido nas augas.

CAPÍTULO 6. Descrición xeral da competencia entre os oxidantes de metano e amonio nunha contorna de escaseza de osíxeno.

As membranas de filtración permiten a retención selectiva de certos compostos polo tamaño de partícula. As células poden reterse xa que o seu tamaño é normalmente máis pequeno que o tamaño poroso da membrana. Este feito foi visto como unha oportunidade para cultivar e colleitar microorganismos especializados de crecemento lento.

Utilizáronse dous reactores de mestura completa axustados en serie, para promover o crecemento de bacterias oxidantes tanto de amonio como de nitrito, respectivamente. Utilizouse como inóculo un cultivo mixto dun lodo procedente dunha

investigación anterior. Establecéronse as condicións operativas, tales como a temperatura ou o tempo de retención de sólidos, co obxectivo de obter os cultivos específicos obxectivo do estudo. Con todo, observouse un rápido crecemento de representantes de commamox. Os que executan a oxidación completa de amonio (commamox) son microorganismos recentemente descubertos que pertencen ao xénero *Nitrospira* e que poden oxidar o amonio ata nitrato con só un microorganismo. Aínda que o cultivo de commamox foi accidental no sistema estudado, demostrouse a capacidade das membranas de enriquecer o lodo neses microorganismos.

LIST OF SYMBOLS

AOB: ammonium oxidizing bacteria

BPC: Biopolymer Clusters [mg/L]

BOM: biological oxygen monitoring

CAS: Conventional Activated Sludge

cBPC: Colloidal fraction of Biopolymer Clusters [mg/L]

CAGR: compound annual growth rate

CAPEX: Capital expenses

CEB: Chemical Enhanced Backwashing

COD: Chemical Oxygen Demand [mg/L]

CSTR: Continuous Stirred Tank Reactor

DIC: Dissolved Inorganic Carbon [mg/L]

DIN: Dissolved Inorganic Nitrogen [mg/L]

DOC: Dissolved Organic Carbon [mg/L]

EPS: Extracellular Polymeric Substances [mgX/L]

GHG: GreenHouse Gas

HF: Hollow Fiber

HRT: Hidraulic Retention Time [time]

IA: Intermediate Alkalinity [mgCaCO$_3$/L]

iWWTP: Industrial wastewater treatment plant

JRC: Joint Research Center

MBR: Membrane Bioreactor

MBBR: Moving Bed Biofilm Reactor

MF: Microfiltration

MLTSS: Mixed Liquor Total Suspended Solids [g/L]

MLVSS: Mixed Liquor Volatile Suspended Solids [g/L]

NOB: nitrite oxidizing bacteria

OLR: Organic Loading Rate [kgCOD/(m^3·d)]

ORR: Organic Removal Rate [kgCOD/(m^3·d)]

PA: Partial Alkalinity [mgCaCO$_3$/L]

PCR: polymerase chain reaction

PE: Polyethylene; population equivalent

PES: Polyethylsulfone

PhD: Philosophy doctor

PLC: Programmable Logic Controller

PP: Polypropylene

RO: Reverse Osmosis

OPEX: Operational expenditures

QAC: quaternary ammonium compounds

SAD: Specific Air Demand: Nm^3/(m^2·h)

SBR: Sequencing Batch Reactor

SMP: Soluble Microbial Products

SRF: Sludge Resistance to Filtration [m/kg]

SRT: Sludge Retention Time [time]

TA: Total Alkalinity [mgCaCO$_3$/L]

TEP: Transparent Exopolymer Particles [mgXG/L]

TDC: Total Dissolved Carbon [mg/L]

TMF: Tertiary Membrane Filtration

TMP: Transmembrane Pressure [Pressure]

TN: Total Nitrogen [mg/L]

TOC: Total Organic Carbon [mg/L]

TSS: Total Suspended Solids [g/L]

UASB: Upload Anaerobic Sludge Blanket

UF: Ultrafiltration

VFA: Volatile Fatty Acids [mg/L]

WWTP: Waste Water Treatment Plant

XG: Xanthan Gum.
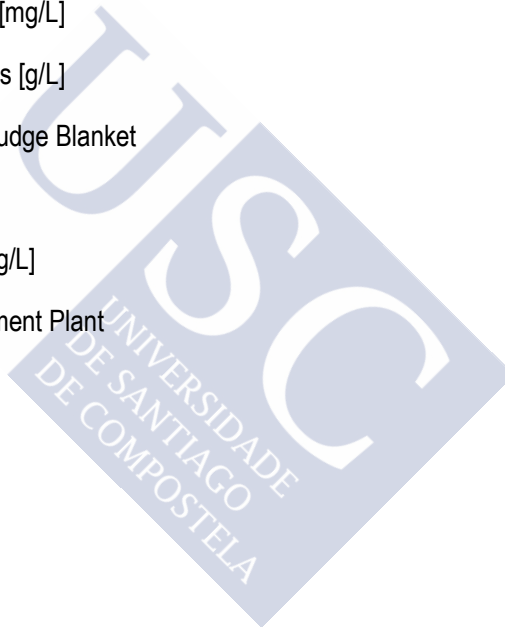
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- **Silva-Teira A.**, Sánchez A., Buntner D., Rodríguez-Hernández L., Garrido J.M. (2017) Removal of dissolved methane and nitrogen from anaerobically treated effluents at low temperature by MBR post-treatment. Chemical Engineering Journal 326 (2017) 970-979.
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- **Silva-Teira A.**, Vázquez-Padín J.R., Reif R., Arias A., Rogalla F., Garrido J.M. (2018) Assessment of a combined UASB and MBR process treating wastewater from a seafood industry at different temperatures [In preparation].

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- Vázquez-Padín J.; **Silva-Teira A.**; Weiler R.; Solís D.; Fernández-González R.; Garrido J.M (2014) Performance of a Hybrid Moving Bed Membrane Bioreactor treating low alkalinity wastewater. IWA World Water Congress and Exhibition. Lisbon – Portugal.

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