UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Ingeniería Química

Advanced systems for biological treatment of high nitrogen-loaded wastewater

Memoria presentada por
Belén Arrojo Arrojo

Para optar al grado de Doctor por la
Universidad de Santiago de Compostela

Santiago de Compostela, Octubre de 2006

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Ramón Méndez Pampín, Catedrático de Ingeniería Química y Anuska Mosquera Corral, Profesora Ayudante de Ingeniería Química de la Universidad de Santiago de Compostela,

Informan:

Que la memoria titulada “Advanced systems for biological treatment of high nitrogen-loaded wastewater”, que para optar al grado de Doctor de Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Doña Belén Arrojo Arrojo, ha sido realizado bajo nuestra inmediata dirección en el Departamento de Ingeniería Química de la Universidad de Santiago de Compostela.

Y para que así conste, firman el presente informe en Santiago de Compostela, el 30 de Octubre de 2006.

Ramón Méndez Pampín Anuska Mosquera Corral

Esta memoria fue presentada el día 16 de febrero de 2007 en el salón de actos de la Escuela Técnica Superior de Ingeniería (ETSE) de la USC ante el tribunal compuesto por:

Dr. José Mario Díaz Fernández de la Universidad de Oviedo.
Dra. Merle K.de Kreuk de la Universidad Técnica de Delf (Holanda).
Dra. Elena Ficara del Politécnico de Milán (Italia).
Dr. Julián Carrera Muyo de la Universidad Autónoma de Barcelona.
Dr. José Luís Campos Gómez de la Universidad de Santiago de Compostela.

Con la calificación de *Sobresaliente Cum Laude y la mención de Doctorado Europeo.*

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“Ricordati quando commenti l’acque
d’allegar prima la sperienza e poi la ragione”

Leonardo da Vinci

Table of contents

Objetivos y Resumen

Las diversas legislaciones, cada vez más exigentes, están reduciendo cada vez más las concentraciones máximas permitidas de contaminantes en los efluentes, lo que está impulsando el desarrollo de sistemas más eficaces y compactos en los cuales se pueda llevar a cabo la eliminación conjunta de materia orgánica y/o nutrientes. Los nuevos sistemas de tratamiento biológico deben ocupar menos espacio, mejorar la retención y producción de lodos, y además deben presentar mejores propiedades para su posterior gestión, logrando así no sólo la mejora de la calidad de los efluentes generados sino también una reducción de costes.

Como alternativa al proceso convencional de lodos activos se propone el empleo de reactores biológicos de lodo granular y sistemas de membranas. El objetivo es lograr una alta eliminación de carbono y nitrógeno en aguas residuales urbanas e industriales y generar, asimismo efluentes tratados aptos para su reutilización.

Estos reactores pueden operar con cargas más elevadas (plantas más compactas) debido al desarrollo de una biomasa agregada en forma de gránulos, con buenas características de sedimentabilidad, lo que posibilita su fácil separación en el propio reactor, siendo posible sustituir los grandes y costosos decantadores secundarios por ejemplo por unidades de membrana externas mucho más pequeñas que permiten mejorar la calidad física, química y microbiológica del efluente y propiciar su reutilización.

Por otro lado, la alta productividad de los microorganismos heterótrofos empleados en los sistemas convencionales de tratamiento de aguas trae consigo la

generación de grandes cantidades de lodos, que suponen un importante aumento en los costes de operación, así como, un problema ambiental. El uso de gránulos en el tratamiento de aguas residuales puede contribuir a la reducción del residuo generado mediante dos posibles vías:

1) Reducción de la cantidad de lodos producidos: Se ha encontrado que la producción de lodos en sistemas granulares era un 30% menor que en sistemas de lodos activos. Este hecho, puede deberse a que los microorganismos que crecen formando gránulos, tienen una mayor proporción de exopolímeros en su composición para mantener su estructura. La producción de dichos compuestos implica la variación del metabolismo de los microorganismos, empleando más energía en este proceso en comparación con los floculantes.

2) Reducción del volumen de lodo: durante el tratamiento del lodo generado la mayor parte de los procesos aplicados se centran en la reducción de su volumen mediante la disminución de su contenido en agua. Dado que el mecanismo de selección de los microorganismos formadores de gránulos en los biorreactores granulares es la velocidad de decantación, dichos gránulos tienen estructuras más compactas y más densas que los flóculos lo que lleva directamente a un menor volumen de lodo. Por otra parte, se ha encontrado que durante la granulación las células aumentaban su hidrofobicidad del 50 al 80%, lo que puede favorecer los procesos de deshidratación del lodo.

Los estudios que se han llevado a cabo hasta la fecha en el desarrollo de biorreactores granulares aerobios han permitido establecer la hipótesis de que la formación de lodos granulares es posible en sistemas operados en ciclos con cortos períodos de alimentación y de sedimentación.

Otros factores que afectan a las características de los gránulos formados son: i) El tipo de efluente tratado, urbano o industrial; ii) Condiciones de estrés, es decir cuanto mayor es el estrés aplicado, menor es el tamaño de los gránulos formados y mayor su densidad mejorando así su sedimentación; iii) Cuanto más lentamente crece la biomasa al degradar un substrato, más densos y pequeños serán los gránulos obtenidos, siendo necesario evaluar el efecto de los sólidos presentes en el efluente tratado; iv) Concentración de oxígeno: bajas concentraciones de oxígeno mejoran la eficacia de eliminación de nitrógeno en los biorreactores

granulares, pero en muchos casos impiden o ralentizan la formación de los gránulos; v) Geometría del reactor (relación altura/diámetro).

En la presente tesis se estudian inicialmente el efecto de la composición del agua residual sobre la formación del gránulo, investigando posteriormente las condiciones hidrodinámicas más propicias para desarrollar los gránulos en condiciones aerobias. El desarrollo y aplicación de sistemas granulares se hace tanto en aguas con una alta relación C/N, a las que se aplica el proceso de nitrificación-desnitrificación, como en aguas con una baja relación C/N, a las que se aplica un proceso avanzado de oxidación anaerobia de amonio (Anammox). Además, se ensayan distintas condiciones de operación con el fin de maximizar la eficacia de eliminación de la materia orgánica y del nitrógeno, para cada tipo de agua residual a tratar, y tipo de lodo granular empleado. El lodo granular producido se cuantifica y caracteriza durante la operación de los reactores.

Los sistemas granulares producen unos efluentes de buena calidad, en términos de contenido en materia orgánica y nitrógeno, pero con concentraciones de sólidos en suspensión que en ocasiones no se ajustarían a los requerimientos más estrictos (menores de 35 mg SS/L), lo que justifica la utilización de sistemas de filtración de membranas como tratamiento complementario.

En los sistemas de membranas se evaluan las mejores condiciones de operación (tipo de membrana, ensuciamiento, protocolos de limpieza, etc.) así como la calidad fisico-química y microbiológica de los efluentes generados para evaluar la posible reutilización de los mismos para uso industrial, agrícola o de riego, tomando como referencia las normas nacionales, comunitarias e internacionales vigentes.

En el **Capítulo 1**, se hace una revisión bibliográfica relacionada con la granulación en condiciones aerobias/anóxicas/anaerobias, que incluye la formación de los gránulos y su comportamiento, los procesos de eliminación biológica de nitrógeno (nitrificación, desnitrificación y Anammox), y la tecnología de membranas.

En los últimos años, la investigación sobre los procesos de granulación ha sido muy amplia. Hasta la fecha, la mayoría de los gránulos obtenidos en

condiciones aerobias se ha llevado a cabo en reactores discontinuos tipo SBR (Sequencing Batch Reactor). En estos sistemas SBR se obtienen gránulos con menos producción de lodos y mayores velocidades de conversión que en los sistemas convencionales de lodos activos. Altas concentraciones de biomasa se pueden obtener en estos sistemas debido a la compacta estructura de los gránulos aerobios. Además esta tecnología se basa en el uso de un reactor único (SBR), en el que se realizan todas las fases de operación: llenado, reacción, sedimentación y vaciado. Este hecho tiene la ventaja de que es necesario disponer de menos espacio y equipos para llevar a cabo el proceso.

Diferentes parámetros afectan a la formación de gránulos aerobios/anóxicos/anaerobios en SBRs como pueden ser: productividades celulares de los diferentes microorganismos, fuerzas de estrés (que dependerán de la hidrodinámica del reactor), selección de la biomasa que se lleva a cabo a través del tiempo de decantación, tipo de sustrato, Demanda Química de Oxígeno (DQO), carga de nitrógeno y concentración de oxígeno.

El proceso Anammox (ANaerobic AMMonium OXidation), se presenta en la actualidad como una alternativa eficaz para eliminar compuestos nitrogenados presentes en aguas residuales con alto contenido de nitrógeno, pero baja concentración de materia orgánica, en comparación con los procesos convencionales de nitrificación/desnitrificación. Recientemente, el número de trabajos enfocados al estudio del proceso Anammox se ha incrementado, sin embargo, pocos de ellos se han encaminado al estudio de los aspectos ingenieriles para llevar a cabo la implantación de dicho proceso a escala real. En la presente tesis se evalúan estos aspectos.

En el **Capítulo 2** se describen los métodos analíticos usados en el trabajo. Esto incluye, tanto los parámetros convencionales de caracterización de aguas residuales (materia orgánica, compuestos nitrogenados, pH, oxígeno disuelto, sólidos y compuestos de carbono), como la caracterización de la biomasa. En este último caso, la caracterización de la biomasa se llevó a cabo utilizando parámetros como: la densidad de los gránulos, el índice volumétrico de lodos y otras técnicas como pueden ser el análisis de imagen, observación con lupa y microscopio y finalmente la microscopía electrónica. La identificación de los

diferentes tipos de poblaciones se realizó usando la técnica de FISH (Fluorescent In Situ Hybridisation).

La técnica del FISH permite la detección selectiva de un microorganismo en particular presente en un lodo biológico conteniendo muchos otros microorganismos diferentes. Este método se basa en el uso de la secuencia específica de la subunidad 16S del ARN ribosomal (ARNr) perteneciente al microorganismo en cuestión. En esta técnica se usan cadenas de oligonucleótidos (ACGT) preparadas sintéticamente y que son complementarias a zonas específicas de la cadena del 16S ARNr que permiten identificar a un microorganismo o grupo dependiendo de la especificidad de la cadena. A estas cadenas sintéticas se les denomina sondas de oligonucleótidos y llevan adherido a su molécula un tinte fluorescente. Estas sondas de oligonucleótidos son muy estables, fáciles de conseguir, relativamente baratas, de fácil manejo, específicas, penetran bien en las células, dan resultados reproducibles y los tintes con los que están marcadas no interfieren en la hibridación. Esta sonda ha de introducirse en el interior de las células que se quieren identificar para lo que se llevan a cabo una serie de procedimientos con el fin de hacer que la pared celular sea permeable a la sonda, y que existan las condiciones ambientales adecuadas para que está se acople a la zona específica del 16S ARNr. Una vez la sonda está unida podemos observar al microscopio de epifluorescencia las células. Aquellas que tengan la sonda dentro “están marcadas” y por tanto emiten fluorescencia cuando se observan al microscopio de epifluorescencia, es decir se ven de color.

Mientras que la composición de las aguas residuales urbanas está bien definida y la concentración de sus componentes no sufre grandes variaciones, no ocurre lo mismo para las aguas residuales industriales. Sus características van a depender del proceso en que se generen, lo que conlleva un estudio específico para cada tipo de agua antes de la aplicación del proceso de tratamiento biológico.

Un factor importante en la dinámica de formación de los gránulos y en su estructura es el tipo de substrato empleado. Por lo tanto, será de interés estudiar la formación de los gránulos y sus características físicas en distintos tipos de aguas residuales, dado que van a condicionar tanto la capacidad de tratamiento del sistema como el post-tratamiento del lodo generado.

En el capítulo 3 se comenzó a estudiar la formación de lodo granular en dos reactores de laboratorio (SBRs), alimentados uno de ellos con agua sintética y el otro con un agua industrial. En este último caso, las aguas residuales procedían de un laboratorio de análisis de muestras de leche de vacuno situado en Galicia (Laboratorio Interprofesional Galego de Análise do Leite, LIGAL). Dichas aguas residuales se originan por la mezcla de tres corrientes principales: aguas fecales y sanitarias, aguas generadas durante los diversos trabajos de análisis realizados, y una tercera corriente que recibe la descarga de todas las muestras de leche que este laboratorio recibe, después de proceder a su control de calidad. Las características del efluente final generado son similares a las de los producidos en las industrias lácteas. Así, la materia orgánica está comprendida entre 5 y 10 g DQO/L, mientras que la concentración de nitrógeno total oscila en torno a 0,20 g N/L. Esta planta dispone de un sistema de tratamiento compuesto por un filtro anaerobio de 12 m^3 y un reactor secuencial (SBR) de 28 m^3, usando para el presente trabajo el efluente del filtro anaerobio.

Los dos reactores SBRs de laboratorio se han operado en condiciones similares durante la mayor parte del período experimental. Sin embargo, en uno de ellos (R1) se ha incluido una fase anóxica al principio del ciclo de operación que dura entre 10 y 30 minutos.

Los dos reactores se han inoculado con el lodo floculento procedente del SBR industrial y se han operado a altas velocidades de carga orgánica y nitrogenada (VCO y VCN) alcanzándose valores de 7 g DQO/(L·d) y 0,7 g N/(L·d). Los porcentajes de eliminación de nitrógeno estuvieron alrededor del 70% en ambos reactores, incluso considerando que uno de ellos operaba siempre en condiciones aerobias. La morfología de los gránulos también fue muy similar y el tamaño de estos estuvo comprendido entre 0,25 y 4,0 mm.

El objetivo del **Capítulo 4** fue obtener gránulos nitrificantes partiendo de los gránulos heterótrofos previamente obtenidos. Para ello, la estrategia a seguir fue disminuir paulatinamente la relación DQO/N, hasta que la DQO fue eliminada totalmente del medio. La estructura de los gránulos se mantuvo a pesar de los cambios en la alimentación y la concentración de sólidos en el efluente se redujo a 10 mg SST/L cuando el acetato se había eliminado de la alimentación.

Otro objetivo de este capítulo fue estudiar el efecto de las diferentes relaciones carbono/nitrógeno (DQO/N) en la alimentación sobre la producción de compuestos nitrogenados en el efluente. Para esto, se ensayaron diferentes relaciones DQO/N (15; 7; 5; 2,5; 1,25 y 0 g/g), obteniendo para todas ellas porcentajes de eliminación de materia orgánica alrededores del 90%. Los cambios en la relación DQO/N tuvieron como resultado la obtención de diferentes compuestos de nitrógeno en el efluente. La eliminación de nitrógeno se llevó a cabo tanto mediante la asimilación de este compuesto por parte de las bacterias para crecimiento, como por procesos de nitrificación-desnitrificación. El predominio de uno u otro mecanismo dependió de la relación DQO/N que se usase en la alimentación.

El siguiente paso (**Capítulo 5**) fue profundizar en los parámetros que influyen en la obtención y el comportamiento de los gránulos. De este modo, se estudió el efecto de las condiciones hidrodinámicas (fuerzas de estrés y configuración del reactor) sobre la formación de gránulos en un SBR con una inusual relación H/D (altura/diámetro) de sólo 2,5. En este sistema, también se estudió la dependencia de la concentración de materia orgánica (carbono orgánico total) en la obtención de gránulos con unas determinadas propiedades físicas.

Las fuerzas de estrés ejercidas sobre la biomasa afectan a la formación de gránulos aerobios/anaerobios y este efecto será diferente dependiendo del tipo de reactor empleado y del modo en que se logra la mezcla en el sistema: agitación mecánica o agitación por gas.

En el **Capítulo 6** se evalúan el efecto de las condiciones de operación hidrodinámicas en el proceso Anammox en diferentes sistemas SBR, tanto con mezcla por agitación mecánica (reactor SBRM) como por flujo de gas (reactores SBRF1 y SBRF2). El reactor SBRM se operó durante más de 200 días a diferentes velocidades de agitación (60-250 rpm) y los reactores SBRF1 y SBRF2 operaron durante más de 100 días a diferentes velocidades de gas (3,53-12,35 cm/min). De esta forma, los reactores se expusieron a diferentes condiciones de estrés y se estudió como estás fuerzas afectaban al comportamiento de los gránulos Anammox. La velocidad de carga nitrogenada a la que operó el reactor estuvo comprendida entre 0,05 g N/(L·d) y 0,3 g N/(L·d), siendo el último valor el

utilizado durante la operación en condiciones estables. El porcentaje de eliminación de nitrito (substrato limitante) fue del 98% durante la mayoría del período operacional. La actividad específica Anammox de la biomasa fue prácticamente constante y estuvo entorno a 0,4 g N/(g VSS·d) para el SBRM y alrededor de 0,35 g N/(g VSS·d) para SBRF1, 2. El diámetro medio de los gránulos fue de 0,64 y 0,75 mm para SBRM y SBRF1, 2, respectivamente.

Los resultados obtenidos indican que hay un valor límite tanto de agitación (180 rpm) como de velocidad de gas (7,39 cm/min) para que el proceso Anammox se lleve a cabo de forma satisfactoria. En el reactor SBRM, cuando fue operado a 250 rpm, la actividad Anammox disminuyó alrededor de un 45%, incrementándose con ello la concentración de sólidos en el efluente a 0,2 g SST/L y la concentración de nitrito a 60 mg N/L. En el caso de los reactores SBRF, cuando se operaron a 9,7 cm/min, la actividad Anammox disminuyó un 85%, y el diámetro medio de los gránulos también decreció en un 30%, además de producirse una acumulación de 70 mg N/L de nitrito.

A pesar de que los sistemas SBR granulares permiten la acumulación de altas cantidades de biomasa dentro del reactor, la calidad del efluente está normalmente limitada por el contenido de sólidos en suspensión. La utilización de postratamientos, como por ejemplo, los sistemas de membranas, permite la mejora en la calidad del efluente tanto en términos de sólidos en suspensión como en calidad microbiológica.

En el **Capítulo 7** se estudió la eliminación de componentes patógenos del agua usando un SBR y una membrana acoplada en serie. El uso de la membrana, no sólo mejoró la calidad del efluente en términos de sólidos en suspensión sino que también en términos microbiológicos. Se observó una eliminación parcial de coliformes fecales y *Escherichia coli* en el efluente del SBR, previo a la filtración por la membrana. La utilización de la membrana aseguró una total eliminación de coliformes en el permeado final. Se eliminó más de un 95% de la materia orgánica, sólidos en suspensión y bacterias coliformes.

La utilización de la tecnología de membranas se presenta como una buena alternativa a los sistemas convencionales para tratar aguas residuales en el caso de requerir un efluente con una alta calidad en términos de sólidos, materia

orgánica, nitrógeno y bacterias coliformes, pudiendo ser apto para su posible reutilización. La reutilización directa de esta agua se podría llevar a cabo en la industria, agricultura o para riego de jardines, para lo cual se comparará la calidad del permeado con los niveles exigidos en la normativa nacional o internacional.

Obxectivos e Resumo

Neste estudio preséntanse diferentes vías relacionadas co tratamento biolóxico das augas residuais con concentracións altas de nutrintes. As instalacións convencionais de tratamento de augas residuais presentan algunhas desvantaxes como poden ser, baixa capacidade de conversión volumétrica, enfocada principalmente a eliminación de compoñentes fácilmente biodegradables e as altas cantidades de lodo producidas. Para poder cumprir cas lexislacións máis estrictas, por exemplo, as relacionadas ca eliminación de nitróxeno e microcontaminantes, requírese o desenrolo de novos procesos e tecnoloxías.

Na presente tese estúdianse as condicións hidrodinámicas máis favorables para desenrolar gránulos aerobios e tamén o efecto da composición da auga residual na formación de gránulos. O desenrolo e aplicación de sistemas granulares levarase a cabo tanto con augas residuais caracterizadas pola súa alta relación Carbono/Nitróxeno (C/N), usando os procesos de nitrificación-desnitrificación como tamén empregando augas con baixa relación C/N, usando neste último caso o proceso avanzado de oxidación anaerobia de amonio (Anammox). Ensaiaranse diferentes condicións operacionais para así maximizar as eficacias de eliminación de materia orgánica e nitróxeno para cada tipo de auga residual empregada. Caracterizarase e cuantificarase tamén o lodo granular producido durante a operación dos diferentes reactores.

No **Capítulo 1**, presentase unha revisión bibliográfica relacionada coa granulación aerobia, anóxica e anaerobia, incluíndo a formación e o

comportamento de gránulos, os procesos biolóxicos de eliminación de nitróxeno (nitrificación, desnitrificacion e Anammox) así como a tecnoloxía de membrana.

Nos últimos anos, a investigación sobre os procesos de granulación aerobia/anóxica/anaerobia foi moi extensa. Ata a data, a maioría da biomasa obtida como gránulos aerobios tivo lugar nos reactores secuenciais descontinuos (SBR). Estes sistemas SBRs son capaces de desenrolar procesos aerobios con menos producción de lodo e maiores velocidades de conversión que nas tradicionais plantas de lodos activos. Debido a súa estructura moi compacta pódense obter altas concentracións de biomasa nestes sistemas e ademais a carga volumétrica pode ser tamén elevada. Ademais, como a tecnoloxía granular se basea en un só reactor (SBR); todas as fases operacionais lévanse a cabo no mesmo reactor: enchido, reacción, decantación e vertido. Este feito implica a necesidade de usar sistemas máis pequenos.

Diferentes parámetros afectan á formación de gránulos aerobios/anóxicos/anaerobios en SBR como por exemplo, o rendemento celular dos microorganismos empregados, as forzas de estres que dependeran das condicións hidrodinámicas do reactor, a selección de biomasa por medio da velocidade de decantación, tipo de substrato, DQO, carga de nitróxeno e concentración de osíxeno.

O proceso Anammox é unha alternativa para eliminar compoñentes de nitróxeno de augas residuais con alta carga nitroxenada e con baixo contido de materia orgánica, en vez dos procesos convencionais de nitrificación/desnitrificación. Recentemente incrementáronse os traballos sobre o proceso Anammox. Sen embargo, non hai moitos traballos relacionados con aspectos de enxeñería para poder levar a cabo o proceso a escala real. Por iso, neste traballo presentarase especial atención a estes aspectos.

No **Capítulo 2** describiranse os métodos analíticos empregados durante este estudio. Esto inclúe, tanto os parámetros convencionais usados para a caracterización de augas residuais (materia orgánica, compostos de nitróxeno, pH, concentracións de carbono e de sólidos), como a caracterización da biomasa. Esta caracterizouse por medio de parámetros como a densidade dos gránulos, o índice volumétrico de lodos, e técnicas como o análise de imaxe dixital, a microscopía

electrónica e a observación con lupa. A identificación das diferentes poboacións presentes na biomasa levouse a cabo empregando a técnica FISH (Fluorescent In Situ Hybridisation).

O primeiro paso foi estudiar a formación do lodo granular en dous reactores secuenciais a escala de laboratorio (SBR), alimentados un deles con auga residual sintética e outro con auga industrial procedente dun laboratorio de análise de mostras lácteas. Ambos reactores operáronse en condicións similares durante a maioría do período experimental. Sen embargo, nun dos reactores (R1) introduciuse unha fase anóxica o inicio do ciclo de operación de entre 10 e 30 minutos de duración. As velocidades de carga orgánica e nitroxenada (VCO e VCN) aplicadas a ambos reactores foron moi altas alcanzándose valores de 7 g DQO/(L·d) e 0,7 g N/(L·d). Os porcentaxes de eliminación de nitróxeno foron do 70% en ambas unidades incluso considerando que R2 se operou sempre en condicións aerobias. Os gránulos obtidos nos dous sistemas tiveron unha morfoloxía similar e o tamaño deles estivo comprendido entre 0,25 e 4,0 mm (**Capítulo 3**).

O obxectivo do **Capítulo 4** foi obter un lodo granular nitrificante partindo do lodo heterótrofo diminuíndo a relación DQO/N ata eliminar completamente a DQO do medio. A estructura dos gránulos mantívose a pesar dos cambios na alimentación e a concentración de sólidos no efluente diminuíu ata 10 mg SST/L cando o acetato foi eliminado do medio da alimentación.

Outro obxectivo deste capitulo foi estudiar o efecto das diferentes relacións carbono/nitróxeno (DQO/N) na alimentación sobre a producción de compostos nitroxenados no efluente. Para isto, ensaiáronse diferentes relacións DQO/N (15; 7; 5; 2,5; 1,25 y 0 g/g), obtendo para todas elas porcentaxes de eliminación de materia orgánica o redor do 90%. Os cambios na relación DQO/N tiveron como resultado a obtención de diferentes compostos de nitróxeno no efluente. A eliminación de nitróxeno levouse a cabo tanto mediante a asimilación deste composto por parte das bacterias para o crecemento, como por procesos de nitrificación-desnitrificación. O predominio dun o outro mecanismo dependeu da relación DQO/N que se empregase na alimentación.

Estudiouse tamén o efecto das condicións hidrodinámicas (forzas de estres e configuración do reactor) sobre a formación de gránulos nun SBR cunha relación H/D (altura/diámetro) de só 2,5. Neste sistema, tamén se estudiou a dependencia da concentración da materia orgánica (carbono orgánico total) na obtención de gránulos con unhas determinadas propiedades físicas (**Capítulo 5**).

No **Capítulo 6** evalúouse ó efecto das condicións de operación hidrodinámicas no proceso Anammox en diferentes sistemas SBR, tanto con mixtura por axitación mecánica (reactor SBRM) como por fluxo de gas (reactores SBRF1 e SBRF2). O reactor SBRM operouse durante máis de 200 días a diferentes velocidades de axitación (60-250 rpm) e os reactores SBRF1 e SBRF2 operáronse durante máis de 100 días a diferentes velocidades de gas (3,53-12,35 cm/min). De esta forma, os reactores expuseronse a diferentes condicións de estres e estudiouse como estas forzas afectaban o comportamento dos gránulos Anammox. A velocidade de carga nitroxenada a que operou o reactor estivo comprendida entre 0,05 g N/(L·d) e 0,3 g N/(L·d), sendo o último valor o utilizado durante a operación en condicións estables. O porcentaxe de eliminación de nitrito (substrato limítante) foi do 98% durante a maioría do período operacional. A actividade específica Anammox da biomasa foi practicamente constante e estivo entorno a 0,4 g N/(g VSS·d) para o SBRM e o redor de 0,35 g N/(g VSS·d) para o SBRF1, 2. O diámetro medio dos gránulos foi de 0,64 e 0,75 mm para SBRM e SBRF1, 2, respectivamente.

Os resultados obtidos indican que hai un valor límite tanto de axitación (180 rpm) como de velocidade de gas (7,39 cm/min) para que o proceso Anammox se leve a cabo de forma satisfactoria. No reactor SBRM, cando se operou a 250 rpm, a actividade Anammox diminuíu o redor dun 45%, incrementándose con elo a concentración de sólidos no efluente a 0,2 g SST/L e a concentración de nitrito a 60 mg N/L. No caso dos reactores SBRF, cando se operaron a 9,7 cm/min, a actividade Anammox diminuíu un 85%, e o diámetro medio dos gránulos tamén diminuíu nun 30%, ademais de producirse unha acumulación de 70 mg N/L de nitrito.

A pesar de que os sistemas SBR granulares permiten a acumulación de altas cantidades de biomasa dentro do reactor, a calidade do efluente está normalmente

limitada polo contido de sólidos en suspensión. A utilización de postratamentos, como por exemplo, os sistemas de membranas, permiten a mellora na calidade do efluente tanto en termos de sólidos en suspensión como na calidade microbiolóxica.

No **Capítulo 7** estudiouse a eliminación de compoñentes patóxenos da auga usando un SBR e unha membrana acoplada en serie. O uso da membrana, non só mellorou a calidade do efluente en termos de sólidos en suspensión senón que tamén en termos microbiolóxicos. Observouse unha eliminación parcial de coliformes fecais e *Escherichia coli* no efluente do SBR, previo a filtración pola membrana. A utilización da membrana asegurou unha total eliminación de coliformes no permeado final. Eliminouse máis dun 95% da materia orgánica, sólidos en suspensión e bacterias coliformes.

Objectives and Summary

In this work, different studies referred to the biological treatment of wastewaters with high nutrients concentration are presented. The conventional wastewater treatment installations have some inherent disadvantages, like the low volumetric conversion capacities, focussed mainly to the removal of easily degradable organic compounds and the high amounts of sludge production. To be able to cope with more stringent regulations, e.g., regarding to nitrogen and micropollutants removal, the development of new processes and technologies is required.

In the present thesis the most favourable hydrodynamic conditions to develop the granules in aerobic conditions will be studied, with the further investigation of the effect of the wastewater composition on the granules formation. The development and application of granular systems will be performed both with wastewater characterized by its high Carbon/Nitrogen (C/N) ratio, using the nitrification-denitrification processes, and also with wastewater having a low C/N ratio, using the advanced processes of anaerobic ammonia oxidation (Anammox). Different operational conditions will be tested in order to maximize the organic matter and nitrogen removal efficiencies for each wastewater studied. The granular sludge produced during the operation of the reactors will be quantified and characterized.

In **Chapter 1**, a literature overview related to aerobic/anoxic/anaerobic granulation, including granules formation and performance, biological nitrogen removal processes (nitrification, denitrification and Anammox) and membrane

technology is presented.

In recent years, the research on aerobic/anoxic/anaerobic granulation has been intensive. So far, most of the biomass obtained as aerobic granules is formed in sequencing batch reactors (SBR). These SBR systems are suitable to perform aerobic processes with less sludge production and higher conversion rates than in conventional activated sludge plants. Because of the compact structure of the aerobic granules, high biomass concentrations can be obtained in these systems and therefore the volume load of these reactors can be high. Since the aerobic granular sludge technology is based on a one-reactor system (SBR); all operational phases: influent feeding, reaction, settling and effluent withdrawal take place in one reactor. This fact involves the necessity of smaller reactor systems.

The formation of aerobic/anoxic/anaerobic granules in SBR is affected by different parameters, e.g. biomass yields of the involved organisms, shear stress depending on the hydrodynamics of the reactor, biomass selection by means of the settling rate, the type of substrate, COD and N-load and oxygen concentration are important parameters.

The Anammox process is an alternative to remove nitrogen compounds from high nitrogen loaded wastewater with low organic matter content, instead of the traditional combined nitrification/denitrification processes. Recently the number of research works focused on the study of the Anammox process has increased. Nevertheless, there are scarce studies related to the engineering aspects to implant this process at full scale. In this work those aspects are evaluated.

In **Chapter 2**, the analytical methods used in this work are described. It comprises the conventional parameters used for wastewater (organic matter, nitrogen compounds, pH, dissolved oxygen, solids and carbon compounds concentrations) and the biomass characterisation. The biomass was characterised by means of parameters such as granules density, volumetric sludge index and techniques such as digital image analysis, electronic microscopy and

stereomicroscope. Identification of the different populations present in the biomass samples was researched by Fluorescent In Situ Hybridisation (FISH).

The first step was to study the granular sludge formation in two laboratory scale sequencing batch reactors (SBR), fed with synthetic and industrial wastewater produced in a laboratory for analysis of dairy products. Both reactors were operated under similar conditions during most of the experimental period. However, an anoxic phase between 10 and 30 min was included at the beginning of every cycle of operation of R1, but not in R2. Organic and nitrogen loading rates (OLR and NLR) applied to both systems were high, up to 7 g COD/(L·d) and 0.7 g N/(L·d). Nitrogen removal efficiency was 70% in both units even considering that R2 was operated always under aerobic conditions. Granules with similar morphology were developed in both systems. Granular size distribution was comprehended between 0.25 and 4.0 mm for both systems (**Chapter 3**).

The objective of **Chapter 4** was to obtain a nitrifying granular sludge from heterotrophic sludge by decreasing the COD/N until COD was completely eliminated. In spite of the changes in the feeding composition the granules maintained their structures and the solids content in the effluent was reduced to 10 mg TSS/L when acetate was removed from the feeding media.

Another objective of this chapter was to study the effect of different carbon to nitrogen ratios (COD/N) in the feeding on the production of nitrogen compounds in the effluent. Different COD/N ratios of 15, 7, 5, 2.5, 1.25 and 0 g/g in the feeding were tested. The COD removal percentage was around 90% during the whole operational period. Changes on the COD/N ratio provoked the presence of different concentrations of nitrogen compounds in the effluent. The N removal percentages obtained in the reactor were up to 55%. Removal of ammonia was carried out by both assimilation and simultaneous nitrification-denitrification processes. The predominance of each mechanism was related to the COD/N ratio in the feeding media.

The effects of hydrodynamic conditions (shear force and reactor configuration) on aerobic granulation are studied in a SBR with an unusual H/D ratio of 2.5. The effect of different carbon to nitrogen ratios (TOC/N) in the feeding on the production of nitrogen compounds in the effluent are also studied.

The dependence of the TOC concentrations in the influent on the granulation process are evaluated (**Chapter 5**).

In **Chapter 6**, the effect of operating hydrodynamic conditions on the Anammox process are studied in SBR where complete mixture was achieved by means of mechanical stirring (SBRM) or gas flow (SBRF1 and SBRF2). The reactor SBRM was operated during 218 days under different stirring speeds (60-250 rpm) and the reactors SBRF1 and 2 were operated for 140 and 110 days respectively under different upflow velocities (3.53-12.35 cm/min). In this way the reactors were exposed to different shear conditions and the stability and performance of the Anammox granules was studied.

The nitrogen loading rate (NLR) fed to the SBR ranged from 0.05 g N/(L·d) to 0.3 g N/(L·d), being the latter the chosen value during stable conditions. The nitrite (limiting substrate) removal percentage was 98% during most of the operational period. The specific Anammox activity of the biomass was practically constant and around 0.4 g N/(g VSS·d) for the SBRM and 0.35 g N/(g VSS·d) for the SBRF2. The average feret diameter of the formed granules was 0.64 mm and 0.75 mm for the SBRM and SBRF1, 2, respectively.

Limit values for the accurate operation of the Anammox granular systems were around 180 rpm and 7.39 cm/min for the SBRM and SBRF respectively. In the SBRM the Anammox activity decreased to 50% when a rotating speed of 250 rpm was tested and the average diameter decreased in 45%, the concentration of solids in the effluent increased to 0.2 g TSS/L and nitrite was accumulated in the reactor up to 60 mg N/L. In the case of the SBRF the Anammox activity decreased to 85% when upflow velocity of 9.7 cm/min was applied and the average diameter decreased in a 30% while nitrite accumulated in the reactor up to 70 mg N/L.

Although granular SBR systems allow the accumulation of relative high amounts of biomass inside the reactors the effluent quality is usually limited by the suspended solids content. Utilization of post treatments, like membrane

systems, allows the enhancement of the effluent quality in terms of suspended solids and presence of coliform bacteria.

Wastewater reclamation was studied by using a lab-scale biological reactor and an external filtration membrane coupled in series (**Chapter 7**). The use of the membrane enhanced the quality of the produced effluent from the biological reactor in terms of suspended solids and presence of indicator bacteria. Partial removal of faecal coliforms and *Escherichia coli* was observed in the effluent of a SBR, previous to filtration by the membrane. The use of the membrane ensures a full removal of the indicator bacteria in the final permeate. More than 95% of the organic matter, suspended solids, and coliform bacteria were successfully removed.

Chapter 1

Introduction

Summary

The conventional wastewater treatment installations have some inherent disadvantages, like the low volumetric conversion capacities, focussed mainly to the removal of easily degradable organic compounds and the high amounts of sludge production. To be able to cope with more stringent regulations, e.g., regarding to nitrogen and micropollutants removal, the development of new processes and technologies is required (aerobic granulation, Anammox process, membrane bioreactor, etc.).

In recent years, the research on aerobic granulation has been intensive. So far, most of the biomass obtained as aerobic granules is formed in sequencing batch reactors (SBR). These SBR systems are suitable to perform aerobic processes with less sludge production and higher conversion rates than in conventional activated sludge plants. Because of the compact structure of the aerobic granules, high biomass concentrations can be obtained in these systems and therefore the volume load of these reactors can be high. Since the aerobic granular sludge technology is based on a one-reactor system (SBR); all operational phases: influent feeding, reaction, settling and effluent withdrawal take place in one reactor. This fact involves the necessity of smaller reactor systems.

The formation of aerobic granules in SBR is affected by different parameters which have been studied in the literature, e.g. biomass yields of the involved organisms, shear stress depending on the hydrodynamics of the reactor, biomass selection by means of the settling rate, the type of substrate, COD- and N-loads and oxygen concentration.

Although aerobic granular SBR systems allow the accumulation of relative high amounts of biomass inside the reactors the effluent quality is usually limited by the suspended solids content. Utilization of post treatments, like membrane systems, allows the enhancement of the effluent quality in terms of suspended solids and presence of coliform bacteria.

1.1. State of the art

Conventional Wastewater Treatment Plants (WWTPs) based on activated sludge technologies present large footprints. This is caused by the relatively poor settling characteristics of activated sludge, resulting in the accumulation of small dry solids concentrations in the aeration tanks and in the low maximum hydraulic loads of secondary sedimentation tanks.

In such a process the bacteria (biomass), which are usually present as flocs, are mixed with the wastewater in a large, aerated basin where the removal of the pollutants takes place In order to treat large amounts of wastewater, large aeration basins are required. The supply of fresh wastewater to the basin and the discharge of treated wastewater from the basin continuously occur. The discharged wastewater is led to the settling tank where the separation of activated sludge from the treated wastewater is carried out by means of gravity settling. The treated wastewater can afterwards be discharged or introduced in a further treatment system. Conventional activated sludge plants produce a lot of sludge. Part of the settled activated sludge is recycled to the reactor basin and a small part purged from the system. Two different units, the aeration basin and the settler, are needed to carry out the wastewater treatment which takes up a lot of ground area. Besides, the settling tank covers a large area, because the settling velocity of the activated sludge flocs is very low (< 1 m/h). Since the available ground area to build the treatment plants is usually limited, there is a need for the development of more compact reactors.

In principle bacteria prefer growing in suspension over growing in a floc, which has again preference over growth in a biofilm or granule. Growing in suspension is the most favourable form because in a floc, and even more in a biofilm or granule, the bacteria experience diffusion limitations for the components involved. Growing as a floc, a biofilm, or a granule only occurs when the bacteria are forced to do so due to environmental conditions (Tijhuis *et al.*, 1994).

In the early 90´s, compact attached growth technologies, for aerobic biological wastewater treatment, were developed in several configurations (immobilised bed, fluidised bed and airlift reactors between others). The main

feature of these continuously operated technologies is their availability to treat high volumetric loads, occasionally without a independent sludge/effluent separation step. The process conditions in airlift reactors are simple and the area requirement is limited due to their small footprints. Because of the large specific biofilm area, the achieved volumetric conversion capacities can be high (Heijnen *et al*., 1990). The main disadvantage of these systems is the relatively high investment costs (Bruin *et al*., 2004).

Granular biomass growth is just a special case of biofilm growth. It has been shown that the structure of biofilms is the net result of biomass growth and detachment processes. Growth of the biomass is mainly influenced by the substrate loading rate and the growth yield. Detachment is mainly influenced by shear force (van Loodsdrecht *et al*., 1995). It has been shown experimentally and by modelling that the right balance between substrate loading and shear can result in smooth and strong biofilms (Tijhuis *et al*., 1995; Kwok *et al*., 1998; Picioreanu *et al*., 2000). Consequently, in the granular sludge SBR of this research a high shear has to be applied on the fast growing heterotrophic activated sludge granules. This can be performed by using a high gasflow rate resulting in high turbulence.

Recent research showed that it is possible to grow granular sludge in a batch-wise operated system without a carrier at high dissolved oxygen concentrations resulting in large biomass concentrations and high volumetric loads (7.5 kg COD/ m^3·d) (Morgenroth *et al.,* 1997; Beun *et al*. 1999; Beun *et al*., 2000; Etterer and Wilderer, 2001). These new granular systems do not need the addition of support material for the biomass attachment as it is the case of the previously mentioned reactors (airlift and so on).

1.2. Granulation systems

1.2.1. Granulation

Technologies developed for wastewater treatment based on the formation of granular biomass include anaerobic and aerobic granulation processes. Anaerobic granulation is relatively well known (Lettinga *et al*, 1980; Liu *et al.,* 2003), but research on aerobic granulation is relatively recent. Many full-scale anaerobic

granular sludge units have been operated worldwide, but only few examples exist of similar units for aerobic granulation (de Bruin *et al*., 2005).

Biomass granulation involves several aspects like: cell-to-cell interactions that include biological, physical and chemical phenomena and so on. Biomass granules are formed through self-immobilization of micro-organisms. These granules are dense microbial consortia packed with different bacterial species and typically contain millions of organisms per gram of biomass. In granular sludge reactors, the large size and relatively high density of individual granules makes them to settle rapidly, which simplifies the separation of the treated effluent from the biomass.

1.2.2. Anaerobic granulation

Anaerobic granulation has been extensively studied and is probably best recognized in the upflow anaerobic sludge blanket (UASB) reactor. Many wastewater treatment plants already apply anaerobic granulation technologies for the removal of organic matter (Alves *et al*., 2000; Hulshoff, 1989; Guiot *et al*., 1992; Schimdt and Ahring, 1996; Liu *et al*., 2003; van Lier *et al*., 2001). The feasibility and efficiency of UASB reactors and their various modifications (the internal circulation reactor) to treat municipal and industrial wastewater have been successfully demonstrated (Lettinga *et al*, 1980; Fang and Chui, 1993; Schmidt and Ahring, 1996). Anaerobic granular sludge comprises a wide variety of micro-organisms. None of the individual species in these microecosystems is capable of completely degrading the influent wastes. Complete degradation of industrial waste involves complex interactions between the resident species. Thus, granular sludge reactors are desirable in wastewater biological treatment processes because a very high number of micro-organisms can be maintained in the bioreactor inside the formed granules. Anaerobic granular sludge has been proved as capable of treating high-strength wastewater contaminated with soluble organic pollutants in compact bioreactors.

The anaerobic granulation technology has some drawbacks like: the need for a long start-up period, a relatively high operation temperature and unsuitability for low-strength organic wastewater. In addition, anaerobic granulation technology is not suitable for the removal of nutrients (N and P) from wastewater.

In order to overcome those weaknesses, research has been devoted to the development of aerobic granulation technologies.

1.2.3. Aerobic granulation

1.2.3.1. Definition

The development of biomass in the form of aerobic granules is being recently under study for its application to the removal of organic matter, nitrogen and phosphorus compounds from wastewater. Aerobic granules present several advantages compared to conventional activated sludge such as excellent settling properties, compact microbial structure, high biomass retention, the ability to withstand shock and toxic loadings, the presence of aerobic and anoxic zones inside the granules to perform different biological processes, etc. (Morgenroth *et al.,* 1997; Beun *et al*. 1999; Peng *et al.* 1999; Tay *et al*. 2001a, b; Lin *et al*. 2003; Liu *et al*., 2003; Yang *et al*., 2003a; Arrojo *et al*., 2004; Liu and Liu, 2006).

The definition of the aerobic granules was proposed at the "*1st IWA-Workshop Aerobic Granular Sludge*" (Munich, 2004) (de Kreuk *et al*., 2005) as:

"Granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs."

The aerobic granules are considered as a stable structure meaning that the values of the sludge volumetric index after 10 and 30 minutes of settling (SVI_{10} and SVI_{30}, respectively) should, for a certain sample, give similar values (Schwarzenbeck *et al.*, 2004),

Other characteristics of aerobic granules are:

- The position of micro-organisms is fixed and it does not change quickly as in an activated sludge floc. The structure of a granule is determined in a matrix of biomass and EPS.
- No carrier material is intentionally involved or added.
- No thickening after settling or rapid settling of the aggregates occurs.

- The minimum size of granules should be around 0.2 mm (de Kreuk *et al*., 2005), in order to be able to separate them form a sludge sample by sieving.

When an aggregate fulfils all characteristics as described above, it can be called aerobic granular sludge.

1.2.3.2. Aerobic granular reactor configuration

So far, most research on aerobic granulation has been conducted in sequencing batch reactors (SBR) (Morgenroth *et al.*, 1997; Beun *et al*., 1999), while no successful aerobic granulation has been observed in continuous culture systems.

Compared to continuous operated reactor systems, the main feature of SBR systems is its cycle operation. Each operation cycle consisted of different phases: filling, aeration, settling, discharging, etc. In SBR systems the settling phase substitutes the performance of the settler in the continuously operated reactors. Furthermore the chosen short settling times are likely to exert a selection pressure on the sludge particles, i.e. only particles that can settle down within the given settling time are retained in the reactor, and otherwise they would be washed out of the system.

The primary design criterium for the SBR is based on the assumption that sludge granules will be formed if flocs are washed out. Sludge granules have a high settling velocity compared to sludge flocs, because granules are denser. So granules require less time to settle than flocs. Therefore the time allowed for settling in the SBR cycle is the main design parameter. A short settling period will eventually select for biomass particles with a high settling velocity (Beun *et al*., 1999).

Because the settling velocity is an important selection criterium, a high H/D ratio (column height/column diameter) is advantageous. A high H/D ratio and the absence of an external settler results in a reactor with a small footprint. Besides, the good settling characteristics allow a short stand-still time for settling, allowing more time for biological purification.

Previous research showed that selection pressure in terms of upflow velocity was a driving force towards successful anaerobic granulation in upflow anaerobic

sludge blanket (UASB) reactors (Hulshoff *et al*., 1998; Alphenaar *et al*., 1993). Although SBR has been extensively used in research on aerobic granulation, the mechanism of aerobic granulation in SBR are not fully understood and especially there is still no consensus on the principal driving force of aerobic granulation.

1.2.3.3. Aerobic granules formation

Aerobic granulation can be regarded as the gathering together of cells through cell-to-cell immobilization to form a stable, contiguous, multicellular association. Evidence shows that aerobic granulation is a gradual process from seed sludge to compact aggregates, further to granular sludge and finally to mature granules (Tay *et al*., 2001a). Obviously, for cells in a culture to aggregate, a number of conditions have to be fulfilled. Existing literature on aerobic granule sludge typically focuses on a few parameters that influence granule formation:

- Substrate composition
- Feast-Famine regime
- Hydrodynamic shear force
- Short settling times
- EPS formation
- Inclusion of divalent cations.

Substrate composition

Aerobic granules have been successfully cultivated with a wide variety of substrates including glucose, acetate, ethanol, phenol and industrial wastewater (Beun *et al*., 1999; Dangcong *et al*.,1999; Peng *et al*., 1999; Tay *et al*., 2001a, 2003a; Tay et al., 2002a; Moy *et al*., 2002; Jiang *et al*., 2002; Yang *et al*., 2003a; Schwarzenbeck *et al*., 2005; Morgenroth *et al.*, 1997; Arrojo *et al*., 2004; Ramadori *et al*., 2006; de Kreuk and van Loosdrecht, 2006). However, granule microstructure and species diversity appear to be related to the type of carbon source. The glucose-fed aerobic granules have exhibited a filamentous structure, while acetate-fed aerobic granules have had a nonfilamentous structure in which rod like species of bacteria predominated. Aerobic granules have been also cultivated with nitrifying bacteria and an inorganic carbon source (Tay *et al*.,

2002b; Tsuneda *et al*., 2003; Mosquera-Corral *et al.,* 2005a; Tsuneda *et al*., 2006). More recently, aerobic granules were also successfully developed in laboratory-scale SBR for treating particulate organic matter-rich wastewater (Schwarzenbeck *et al*., 2005). From this research it can be stated that almost any wastewater with enough biodegradable organic matter content is suitable to be treated in an aerobic granular SBR. Special attention must be paid to specific contaminants which could affect the activity of the system.

Feast - Famine regime

The SBR reactors are operated in sequencing cycles of feeding, aeration, settling and discharging of supernatant. In SBR systems, the aeration period actually consists of two periods: a degradation period in which the substrate is depleted to a minimum, followed by an aerobic starvation period in which the external substrate is no longer available. Thus, it is likely that micro-organisms in SBR systems are subjected to a periodic feast and famine regime, called periodic starvation (Tay *et al*., 2001a). It was proposed that under the periodic feast-famine conditions, bacteria become more hydrophobic and high cell hydrophobicity facilitates microbial aggregation (Tay *et al.,* 2001a; Bossier and Verstraete 1996; Liu *et al*., 2004a), meaning that this periodic regime in SBR systems acts as a kind of microbial selection pressure. When bacteria are subjected to a periodic feast-famine regime, microbial aggregation could be an effective strategy for cells against starvation. However, more recent research showed that aerobic granules could not be successfully developed if the settling time in SBR was not properly controlled, even though a periodic feast-famine regime was present (Qin *et al*., 2004a,b). Negative effects of nutrient starvation on the surface properties of aerobic granules in terms of cell hydophobicity and the content of extracellular polysaccharides were also observed (Zhou, 2004). In addition, when the starvation time in SBR was reduced from 3 h to below 30 min, no significant impact on aerobic granules was observed. This may imply that the periodic feast-famine regime could favour aerobic granulation, but so far there is no solid experimental evidence to show that starvation acts as an inducing force of aerobic granulation in SBR.

Hydrodynamic shear force

Evidence shows that a high shear force favours the formation of aerobic granules and granule stability (Shin *et al*., 1992; Tay *et al*., 2001a; Liu ant Tay, 2006; Iaconi *et al*., 2006). It was found that aerobic granules could be formed only above a threshold shear force value in terms of superficial upflow air velocity above 1.2 cm/s in a column SBR, and more regular, rounder, and compact aerobic granules were developed at high hydrodynamic shear force (Tay *et al*., 2001a; Wang *et al.,* 2005). The density and strength of the granules were also proportionally related to the applied shear force (Tay *et al*., 2003b). These observations may imply that the structure of aerobic granules is mainly determined by the hydrodynamic shear force present in a bioreactor. However, extracellular polysaccharides can mediate both cohesion and adhesion of cells and play a crucial role in maintaining the structural integrity in a community of immobilized cells. Tay *et al*. (2001a) reported that the production of extracellular polysaccharides was closely associated with the shear force and the stability of aerobic granules was found to be related to the production of extracellular polysaccharides (Tay *et al*., 2001c). The extracellular polysaccharides content normalized to protein content, increased with the shear force stimulated bacteria to secrete more extracellular polysaccharides. In fact, shear force-induced production of extracellular polysaccharides has been observed in biofilms (Ohasshi and Harada, 1994). Consequently, the enhanced production of extracellular polysaccharides at high shear can contribute to the compact and stronger structure of aerobic granules. Effects of shear on micro-organisms and aggregates have been discussed further elsewhere (Chisti, 1999).

Tay *et al*. (2004) found how superficial upflow air velocity and type of carbon source affected the stability of aerobic granules in a Sequencing Batch Airlift Reactor. At a low superficial velocity, an outbreak of filamentous micro-organisms was observed that gave rise to a poorly settling sludge and eventual biomass washout. When a higher superficial velocity was used, granules with significantly improved settling characteristics were obtained.

In most discussions about shear, the difficulty of measuring shear plays a role as well as the oxygen concentration. In most cases both parameters are related,

meaning that when low superficial velocities are tested low dissolved oxygen (DO) concentrations are reached and, as a consequence, no stable granular biomass can be obtained due to DO limitation.

Short settling times

Strategies to limit the amount of flocs in an aerobic granule system include the use of short settling and discharging times. In a SBR, wastewater is treated in successive cycles, each lasting a few hours. At the end of every cycle, the biomass is settled before the effluent is withdrawn. Sludge that cannot settle down within given settling time could be washed out of the reactor through a fixed discharge port. Basically, a short settling time preferentially selects for the growth of good settling bioparticles. Thus, the settling time exerts a major hydraulic selection pressure on the microbial community. Qin *et al.* (2004a, b) studied the effect of settling time on aerobic granulation in SBR systems and found that aerobic granules were successfully cultivated and became dominant only in SBR operating at a settling times of less than 5 min, while a mixture of aerobic granules and suspended sludge developed in SBR run at longer settling times. In aerobic granulation research, a short settling time has been commonly employed to enhance aerobic granulation in SBR (Jiang *et al*., 2002; Lin *et al*., 2003; Liu *et al*., 2003; Yang *et al*., 2003b; Wang *et al*., 2004; Hu *et al*., 2005). In fact, at a long settling time, poorly settling sludge flocs cannot be effectively withdrawn; and they may outcompete granule-forming bioparticles. As a result, aerobic granulation could fail in SBR run at longer settling times. This seems to indicate that aerobic granules can form only at short settling times below a critical level being the settling time a decisive factor in the formation of aerobic granules in SBR.

It was recognized that the selection pressure imposed by short settling times should be more important in fully aerobic systems, but in anaerobic-aerobic systems with phosphate accumulating organisms (PAOs), the settling criteria seemed less important because of the inherent tendency of PAOs to aggregate.

EPS formation

Literature reports conflicting data regarding the function of the extracellular polymeric substances (EPS) on the formation of aerobic granules (de Kreuk *et al*.,

2005). Some researches indicated that EPS content increases with granulation, that there exist differences in loosely bound and tightly bound EPS and that within the granule structure insoluble versus soluble polysaccharide gradients occur.

The EPS could be the glue between the micro-organisms present in an aggregate and EPS may have important functions with respect to cell metabolism. It has been proposed that exo-enzymes are often an integral part of the EPS enabling cells to get access to substrate of larger molecule size and that EPS could be important to protect cells against toxic substances because of its absorptive capacity. van Loosdrecht *et al.* (2005) agree on the importance of EPS and that more research is needed before conclusions about the role of EPS can be drawn.

Inclusion of divalent cations

Finally another parameter, possibly influencing the granule formation, is the addition of divalent cations such as iron and calcium to help the granule formation. Tsuneda *et al.* (2005) suggested that high concentrations of cations might increase the rate of granule formation and might influence the stability of the reactor system. Tsuneda *et al.* (2006) obtained nitrifying granules using a industrial wastewater which container a high concentration of ammonium and inorganic salts such as sodium chloride and sodium sulphate.

Jiang *et al.* (2003) reported that addition of Ca^{+2} accelerated the aerobic granulation process. With addition of 100 mg Ca^{+2}/L, the formation of aerobic granules took 16 days compared to 32 days in the culture without Ca^{+2} additions. The Ca^{+2} augmented aerobic granules, which also showed better settling and strength characteristics and had higher polysaccharides content. It has been proposed that Ca^{+2} bind to negatively charged groups presents on bacterial surfaces and extracellular polysaccharides molecules and thus acts as a bridge to promote bacterial aggregation.

All different parameters that were discussed seemed to play a role in the granule stability. There was no concluding agreement on which of these parameters is the crucial one and most probably it is a combination of all. Results obtained from aerobic granulation results must be compared to those obtained

from bulking sludge in order to be able to better understand the granulation procedure and the main parameters involved.

1.2.3.4. Selection by settling rate

Different mechanisms important for aerobic granulation have been described in the literature, e.g. yields of the involved organisms, shear and selection by settling rate.

Selection of granules from a mixture in a SBR can be easily based on the difference in settling velocity between the granules (fast settling biomass) and the filaments and flocs (slow settling biomass). Biomass granules, filaments and flocs were present as a mixture in the reactor. Selection of the biomass granules from the mixture occurred during the settling period (Fig 1.1). The settling length is chosen to guarantee that particles with a settling velocity larger than 10 m/h are effectively retained in the reactor. The rest of the biomass does not settle fast enough and will be taken out with the effluent (Beun *et al*., 2002).

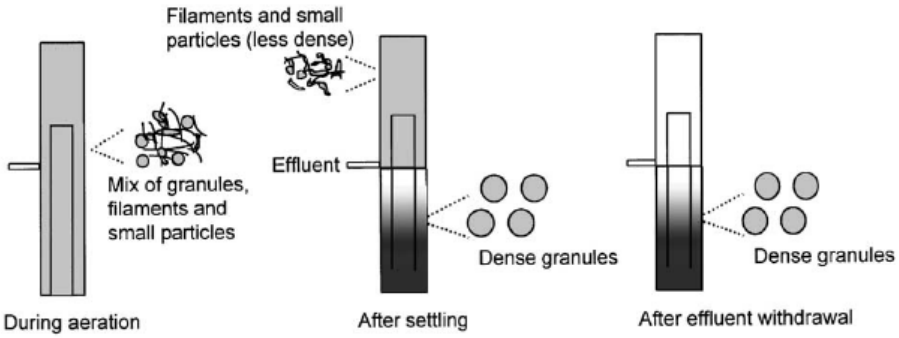


Figure 1.1. Selection of well settling granules in an aerobic granular SBR (Beun *et al*., 2002).

1.2.3.5. Storage of substrates

Since the SBR systems are discontinuously fed reactors, the sludge present in these units experiences periods with external substrate availability (feast period) and periods without external substrate availability (famine period). Under these conditions the micro-organism are able to accumulate substrate as internal storage

products in their cells like glycogen, lipids or polyhydroxyalkanoates (PHA). They can be used by the micro-organisms to survive famine periods and probably also to regulate their growth rate (Zevenhuizen and Ebbink, 1974). Glycogen is usually formed when sugar are present in the liquid. The role of lipids as storage product is unclear. Polyhydroxy butyrate (PHB) is the most dominant storage polymer as it is directly formed from the central metabolite acetyl-CoA (Doi, 1990). Although the presence of storage polymers in activated sludge has often been reported in literature (Zevenhuizen and Ebbink, 1974; Doi *et al*., 1992), only since recently it is generally accepted that they play an important role in especially fed activated sludge processes (van Loosdrecht *et al*., 1997) which could be somehow related to the selection for granule forming micro-organisms. Although recent research indicated that filamentous bacteria and bacteria forming aggregates have similar ability to store PHB (Martins *et al*., 2003).

The knowledge of the PHB metabolism in activated sludge is however limited. In the past, research was mainly focused on the metabolic pathway of PHB formation and degradation (Dawes and Senio, 1973; Doi, 1990; Steinbuchel, 1996), on the enzymes involved in the metabolic reaction steps (Dawes and Senio, 1973; Haywood *et al*., 1989; Anderson and Dawes, 1990; Steinbuchel, 1996), and on the possibilities of industrial production of bacterial PHB (Byrom, 1987; Lee, 1996; Choi and Lee, 1997; van Wegen *et al*., 1998).

PHB exists in the cytoplasm of the cells as granules surrounded by a membrane. The PHB granules have typical diameters of 0.2 to 0.5 μm. It has been found that in most micro-organisms the formation and degradation of PHB occur via a cyclic metabolic pathway (Figure 1.2) (Dawes and Senior, 1973; Doi, 1990).

Acetyl-CoA is the central metabolite in the formation and degradation of PHB. Since acetate is one of the most important substrates in wastewater, this compound is used as a model substrate. Acetate is taken up by the micro-organisms and converted into acetyl-CoA.

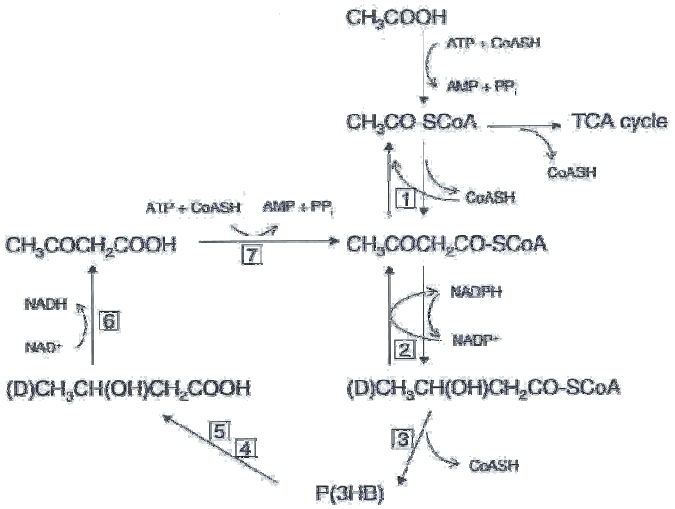


Figure 1.2. Cyclic metabolic pathway of PHB (Dawes and Senior, 1973; Doi, 1990).

In a dynamic system, storing substrate as PHB and thus balancing their growth rate gives these micro-organisms a competitive advantage compared to micro-organisms a competitive advantage compared to micro-organisms that can not store substrate. Bacteria that are not able to store substrate can grow only during the period with external substrate available. During the famine period they can not grow and are even subjected to starvation. In a wastewater treatment system where the sludge usually experiences periods with and without external substrates available, the bacteria that are able to store substrate will finally outcompete the ones that can not store.

More insight into the kinetics and stoichiometrics of PHB production and consumption is needed.

1.2.3.6. Removal of N- containing compounds in the granule

Wastewaters, especially those originating from industries, usually contain ammonium and organic matter. The SBR, containing granular sludge, favours the removal of N-Compounds from wastewater via simultaneous nitrification and denitrification (Beun *et al*., 2001; Mosquera-Corral *et al*., 2005a; Kishida *et al*., 2006). The distribution of autotrophic and heterotrophic biomass in the granules plays an important role (Figure 1.3) (Beun *et al*., 2001).

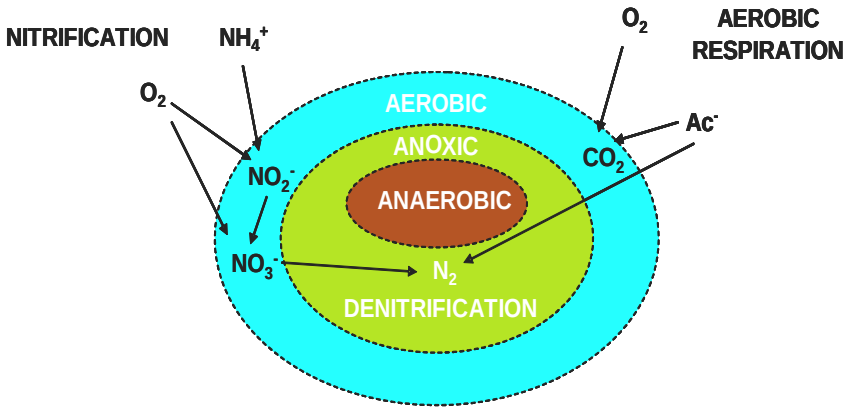
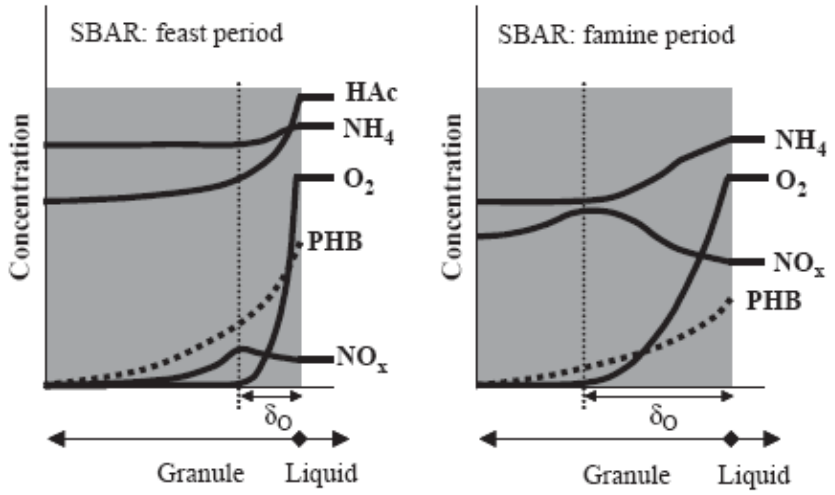


Figure 1.3. Processes combination into the granule.

Since the reactor is sequentially fed, feast and famine periods exist. In the feast period (Fig 1.4a) the concentration of external organic carbon (for example, acetate) is high. This substrate will therefore diffuse into the granules completely. Dissolved oxygen (DO) will have a much smaller penetration depth because it will be consumed very rapidly by autotrophic and heterotrophic organisms in the outer layers of the granules. In the feast period DO is used for nitrification, for aerobic conversion of acetate, and for aerobic biomass growth. Since the autotrophic micro-organism need DO, they tend to be located where this is available. In the case of granular sludge, it is present in the outermost layer of the granules. The autotrophic organisms convert NH_4^+ into NO_3^-. The formed NO_3^- will diffuse towards the centre of the granules, but also towards the liquid phase surrounding the granules. In the centre of the granules acetate can be stored anoxically (using NO_3^-) as PHB by the heterotrophic organisms. In the famine period (Fig 1.4b) the DO penetration depth is larger than in the feast period since its concentration in the liquid is higher. In the centre of the granules NO_3^- is present. The stored PHB in the centre of the granules can be used as organic carbon source for the denitrification. Aerobic conversion of PHB occurs, and nitrification as long as there is NH_4^+ present.



A) B)

Figure 1.4. Theoretical concentration profiles of acetate, PHB, NO_3^- and O_2 during the feast (A) and the famine (B) period in a SBR (Mosquera-Corral *et al*., 2005b)

1.2.3.7. Pilot research in aerobic granular sludge

A large pilot research project based in aerobic granular technology has been started up in The Netherlands in order to demonstrate the applicability of this technology for the treatment of municipal wastewater. Several operational philosophies were tested to learn at which conditions granulation occurs with municipal wastewater as a substrate. Fast formation of granules was observed under conditions of extensive COD-removal, extensive biological phosphate removal and low nitrate effluent concentrations. The potential of the technology is very promising since complete granulation with municipal wastewater as substrate was shown and extensive nutrient removal seems well feasible (de Bruin *et al*., 2005).

The feasibility study showed that the aerobic granular sludge technology seems very promising. Based on total annual costs a GSBR (Granular sludge Sequencing Batch Reactors) with pre-treatment and a GSBR with post-treatment prove to be more attractive than the reference activated sludge alternatives. A sensitivity analysis shows that the GSBR technology is less sensitive to land price and more sensitive to rain water flow. Because of the high allowable volumetric

load the footprint of the GSBR variants is only 25% compared to the references. However, the GSBR with only primary treatment cannot meet the present effluent standards for municipal wastewater, mainly because of exceeding the suspended solids effluent standard caused by washout of not well settleable biomass. (de Bruin *et al*., 2004)

Moreover, municipal wastewater treatment plants (WWTPs) are going to be faced with more stringent effluent standards. In general, activated sludge plants will have to be extended with a post treatment step (e.g. sand filtration) or be transformed into a Membrane Bioreactor. In this case a GSBR variant with primary treatment as well as post treatment can be attractive alternatives.

In case of the full scale plant (de Bruin *et al*., 2005) (Figure 1.5) the start-up period required more time than anticipated and the start-up has not been completed fully yet. Granulation is expected to continue, resulting in larger granules. Although the research with this full scale plant is more or less in the initial stage and many questions are unanswered, the potential of the technology is very promising since complete granulation with municipal wastewater as substrate was shown and extensive nutrient removal seems well feasible.

Figure 1.5. Photo pilot plant

Tay (Tay *et al.*, 2005) also studied the development of aerobic granules in a pilot-scale sequencing batch reactor seeded with aerobic granules precultivated in a small column reactor. It was observed that the seed granules disintegrated in the first few days of operation, but the disintegrated biomass would re-form stable granules latterly. On the contrary, the seed granules could be successfully maintained in the laboratory-scale reactor without significant disintegration. The different hydrodynamic patterns encountered in the pilot and lab-scale reactors might be the reason for the observed phenomena. Factors such as reactor diameter and wall effect, as well as size and placement of air diffusers, would influence the hydrodynamic conditions in the reactors, which in turn determine the properties of the granules.

1.3. Biological nitrogen removal processes

1.3.1. Nitrification and denitrification

Conventional biological nitrogen removal from wastewater is performed in a combined process of nitrification (conversion of NH_4^+ into NO_3^-) and denitrification (conversion of NO_3^- into N_2 gas). Typical continuous flow single sludge systems are the oxidation ditch in which "simultaneous" nitrification and denitrification take place, and pre-denitrification or post-denitrification process schemes are used. Nitrogen removal can also be performed in discontinuous processes (Irvine *et al*., 1983; van Benthum *et al*., 1998). In the aerobic periods nitrification takes place in a two-step process according to two consecutive reactions (eq. [1.1] and [1.2]):

$$NH_4^+ + 3/2\ O_2 \rightarrow NO^-_2 + H_2O + 2\ H^+ \qquad -\Delta G_{298} = 240\text{-}350\ kJ/mol \qquad [1.1]$$

$$NO^-_2 + 1/2\ O_2 \rightarrow NO^-_3 \qquad -\Delta G_{298} = 65\text{-}90\ kJ/mol \qquad [1.2]$$

Ammonium and nitrite are the electron donors and carbon dioxide is the carbon source. The nitrifying bacteria are autotrophic, so they have a slow growing rate. The alkalinity of the wastewaters must be enough to maintain the pH in the optimum interval for nitrification, because 7.13 g of alkalinity as $CaCO_3$ is consumed by 1g of NH_4^+-N oxidized to nitrate (Suthersand and Ganczarczy, 1986). The effect of environmental factors on autotrophic nitrification has been

studied extensively (Strenstrom and Poduska, 1980; Painter and Loveless) and dissolved oxygen has been identified as being of great importance. The oxidation of ammonia in aerobic conditions can produce N_2O as intermediate and this production is stimulated by low DO concentrations (Poth *et al.*, 1985) by the presence of nitrite (Anderson and Levine, 1986; Gejlsbjerg *et al.*, 1998) and by the presence of organic matter (Bock, 1992). In the literature there is a wide range of data about the quality and quantity of N-oxides produced during nitrification (Goreau *et al.*, 1980; Tortoso and Hutchinson, 1990; Sutka *et al*., 2006).

In the anoxic periods denitrification takes place. Nitrate is converted into nitrogen gas. For acetate as carbon source the reaction is (eq. [1.3])

$$5CH_3COOH + 8\ NO_3^- \rightarrow\ 2CO_2 + 4N_2 + 8HCO_3^- + 6\ H_2O \qquad [1.3]$$

Nitrate and nitrite replace oxygen for microbial respiration. Denitrification requires an organic compound as carbon and energy source. This compound is generally the organic substrate present in the raw wastewater.

The efficiency of the use of carbon substrate for denitrification is expressed as the COD/N ratio. Generally this is taken as the ratio in the influent. Here we will use this ratio in a more defined way: the mass of COD used per mass of N denitrified. If no growth occurs this ratio would be 2.86 kg substrate COD/kg N (eq. [1.3]), which can be considered as the theoretical minimum. Due to growth this ratio will normally be around 4 kg substrate COD/kg N. For wastewater treatment often even higher observed values are reported (Henze, 1991).

Different intermediates (N_2O and NO) can be also accumulated during the denitrification process due to the type, concentration of substrate and operational conditions (temperature, pH, HRT, SRT) (Stüven and Bock, 2001; Butler *et al*., 2005; Shaw *et al*., 2006) or due to the presence of toxic compounds (Garrido *et al*., 1998). The presence of low DO concentrations during denitrification also causes the accumulation of N_2O (Schulthess *et al.*, 1994).

1.3.2. Anammox process

The Anammox (Anaerobic AMMonium Oxidation) process consists of the oxidation of ammonium with nitrite as the electron acceptor. Broda (1977) first described this process as a microbial metabolism allowed by equilibrium thermodynamics but not found in nature. Later, in several studies (Mulder, 1992; Mulder *et al.,* 1995) describing the "Anammox" process, ammonium was found to disappear under anoxic conditions and the first identified Anammox organism was named *Candidatus* "Brocadia Anammoxidans". Nowadays different Anammox organisms have been detected by PCR, phylogenetic analysis or FISH in both wastewater treatment and natural systems where nitrogen losses occurred: *Candidatus* "Kuenenia stuttgartiensis", *Candidatus* "Scalindua brodae", *Candidatus* "Scalindua wagneri" and *Candidatus* "Scalindua sorokinii" (Schmid *et al.*, 2003; Kuypers *et al*., 2003).

Anammox process is an alternative to remove nitrogen compounds from high nitrogen loaded wastewater characterized by low organic matter contents, instead of the traditional combined nitrification/denitrification processes. This process consists of the anaerobic oxidation of ammonia (van de Graaf *et al*., 1995,1996) using nitrite as electron acceptor according to the stoichiometry described by Strous *et al.* (1999) (eq. [1.4]). This process allows, in the case of nitrogen removal, the saving of oxygen supply and organic matter compared to nitrification/denitrification processes.

$$NH_4^+ + 1.31\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \quad [1.4]$$

Recently the number of research works focused on the study of the Anammox process has increased. Some of them applied to the study of the metabolic pathways of the process (Schmidt *et al*., 2002; Strous *et al*., 2002) or to the identification of Anammox micro-organisms (Schmidt *et al*., 2001; Mohan *et al*., 2004).

The presence of Anammox organisms has been detected in several types of wastewater treatment processes (Strous *et al*., 2002; Jetten *et al*., 1997, 1999). For example, Anammox activity has been observed in a rotating biological contactor

treating ammonium-rich leachate (Helmer *et al*., 2001; Egli *et al*., 2001), a trickling filter treating wastewater (Schmid *et al*., 2000), fixed-and fluidised-bed reactors and SBRs treating a synthetic medium (Strous *et al*., 1997a,1998).

Application of the Anammox process is limited by the availability of Anammox biomass. The isolation and enrichment of Anammox from a mixture of bacterial populations requires the optimisation of conditions favouring the Anammox process, while limiting the growth of any other kind of microbial population. In particular, since the Anammox process is anaerobic, the exclusion of oxygen is essential especially during the start-up of reactors.

From the point of view of the industrial application the principal disadvantage of this process relies on the presence of different inhibitors (Strous *et al*., 1997b; Dapena-Mora *et al*., 2006a,b) and the slow growth rate of the Anammox micro-organisms (doubling times in the range of 11 - 15 days (Strous *et al*., 1999; Dapena-Mora *et al*., 2004a). For this reason long starting up periods are needed to achieve stable operation conditions. On the other hand, this slow growth rates also imply the operation of systems with high biomass retention properties to maintain high Solids Retention Times (SRT). When these conditions are achieved biomass concentrations in the reactor are high enough to treat relative high nitrogen loading rates. The Sequencing Batch Reactor (SBR) systems have been found to be appropriate to fulfil these conditions according to Strous *et al*. (2002) and van Dongen *et al.* (2001).

Dapena-Mora *et al.* (2004a, 2004b) went further and showed that the SBR is a suitable system to grow Anammox biomass in form of granular sludge. When stable granular sludge is produced it is retained inside the reactor easier than the flocculent sludge due to its better settleability characteristics. Since the Anammox biomass can grow forming granules it is important to have favourable conditions for granulation in order to obtain a stable Anammox population.

Another advantage of the SBRs is the feasibility to change the operational strategy to optimize the efficiency (Humpherys and Banks, 1995) and the easy control of the process (Andreottola *et al.*, 2001). Despite the fact that the SBR is a suitable system to grow Anammox bacteria (Strous *et al*., 1998), it still may present eventual flotation and biomass wash-out problems (Wilderer *et al*., 2001).

Dapena-Mora *et al*. (2004c) improved the capacity to retain biomass of an Anammox SBR by changing the distribution of the SBR operational cycle. The addition of the mixing period in the operational cycle provoked an enhancement in the biomass retention inside the reactor.

1.4. Membrane Bioreactor

With the increased worldwide pressure on water resources, effluent recycle and reuse are developing for irrigation, reclamation and agriculture, as well as for both indirect and direct potable water supply (Côté *et al*., 1997). Until recently, the approach has consisted of providing advanced treatment to a secondary effluent to meet the standards for reuse. For irrigation this treatment complement may be limited to filtration and disinfection. For groundwater recharge, the treatment complement normally involves reverse osmosis (RO) and may become rather complex.

Current and impending legislation in the EC on wastewater effluent discharge has led to the need for enhanced treatment processes capable of removing high percentages of BOD, suspended solids, nitrogen, phosphorus and bacteria (Council Directive, 1975; 1991).

In the field of granulation systems the treatment efficiency is usually limited by the difficulties in separating suspended solids. Sequencing batch reactor (SBR) processes offer several advantages over other types of activated sludge reactors. In particular, the hallmark of SBR design is its inherent flexibility of cyclic phasing. The cycle format can be easily modified at any time to offset changes in process conditions, influent characteristics or effluent objectives (Epa, 1999; Pavelj *et al*., 2001; Pochana and Keller, 1999; Kang *et al*., 2003). However, SBR has a potential risk in that poor clarification and a turbid effluent are associated with it.

Combining a membrane process with an SBR system provides procedural advantages for both processes. The use of membranes can reduce the length of the operational cycle since membrane separation requires no settling and clear-water can be extracted even during the mixing time. In addition, the separation of biological sludge by means of a membrane leads to complete retention of biomass

resulting in a high mixed liquor suspended solids concentration. This allows a very high treatment capacity for a membrane-coupled sequencing batch reactor (MSBR) (Krampe and Krauth, 2000). In a MSBR system, it would be very important, and also difficult to select the most appropriate SBR phase in which membrane filtration could be performed at its best. The reason for this relies on the many types of SBR systems currently in use which operate in different operation modes such as: continuous influent/time based, non-continuous influent/time based, volume based, and intermittent cycle system, and various other system modifications (Banas *et al*., 1999; Schleypen *et al*., 1997; Ketchum, 1997; Irvine *et al.,* 1997). Although combination of membrane filtration and SBR should be reengineered to get a MSBR of good performance, the efficiency of membrane process in MSBR would certainly be dependent on the physicochemical and biological conditions of each SBR phase as well as the type and cycle format of the SBR.

In the past 20 years, the membrane bioreactor (MBR) process has been studied in numerous ways. The main goal of these studies was focused on exploring the feasibility of the MBR process and methods for process enhancement. Emphasis was on effluent quality achieved, higher sludge loading, system compactness, water re-use, and the potential of "zero" sludge growth.

In an MBR solid/liquid membrane filtration occurs either within the bioreactor (submerged configuration, Fig 1.6a or externally through recirculation Fig 1.6b) subject to a pressure drop across the membrane generated by either hydraulic head or a fitted pump. The ultratiltration (UF) or microfiltration (MF) membrane separated the retained (or rejected) materials, principally solids and including micro-organism, from other material largely through sieving, allowing the water and most solute species to pass through the membrane. The membranes typically used in such applications have a pore size between less than 0.1 μm and 0.45 μm, which positions them between ultrafiltration and microfiltration (Figure1.7). These systems are described in greater details in the literature (Yamamoto *et al*., 1989; Chiemchaisri and Yamamoto, 1994; Mourato, 1996; Côté *et al*., 1997; Ueda *et al*., 1997; Buisson *et al*., 1998; Yoon *et al*., 2004).

The advantages offered by MBRs over the conventional activated sludge process (ASP) include a small footprint and reduced sludge production. Submerged MBRs take only half the land area of a conventional ASP, and sludge production in similarly approximately halved (Mayhew and Stephenson, 1997). Since sewage sludge disposal contributed significantly to overall operating costs, there are significant potential benefits in reducing its production (Gander *et al.*, 2000).

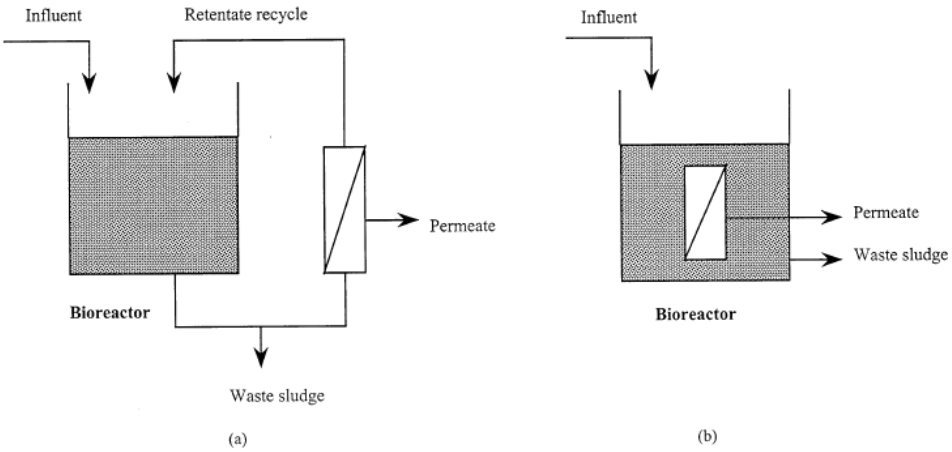


Figure 1.6. a) Side-stream MBR with a separate filtration unit with retentate recycled back to bioreactor; b) submerged MBR: filtration unit integrated into the bioreactor.

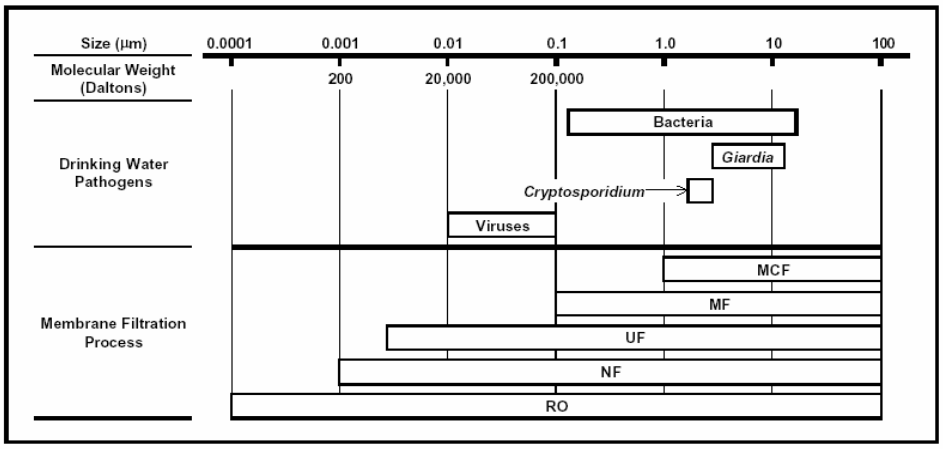


Figure 1.7. Filtration processes versus pore sizes.

1.4.1. Membrane performance

The performance of a given membrane process is represented by:

- The rejection, normally expressed as a function of the ratio of the respective concentrations of the target contaminant in the feed and the permeate product (or filtrate).
- The specific permeate flux (flux per unit pressure) or permeability, sometimes expressed as the hydraulic resistance (inverse product of specific flux and viscosity), and the transient behaviour of this parameter.

Rejection

Rejection is the removal of particles, including biological and non-biological colloids and macromolecules, by sieving or adsorption. One of the main drivers for MBR technology is the ability of the systems to disinfect, resulting in an effluent free from pathogenic micro-organisms. This is an important consideration when discharge is to bathing waters (Council Directive, 1976), or if the water is to be reused. Rejection of both bacteria and viruses by both MF and UF membranes is known to be significant (Kolega *et al*., 1991; Chiemchaisri *et al*., 1992; Judd and Till, 2000; Madaeni *et al*., 1995). Chiemchairsi *et al.* (1992) showed that membranes with 0.03 and 0.1 μm pore size attained the same log reduction of coliphage viruses, and that improved rejection occurred with time owing to the build-up of the dynamic membrane. Madaeni *et al.* (1995) showed *poliovirus* removal to be enhanced by the addition of *E.Coli* to the viral culture in an MF process, possibly because of increased partial pore blocking (0.22 μm) but more likely to be caused by adsorptive retention of the virus on the larger *E.Coli* bacteria.

Different authors (Ueda and Hata, 1999; Ueda *et al*, 1996, 1997; Kishino *et al*., 1996) have reported in several studies that more than 90% of organic matter, suspended solids, and coliform bacteria were successfully removed from a domestic sewage using different reactors with membranes.

Permeate flux

Permeate flux decline is influenced by a number of factors relating to the feed water (composition), the membrane (element geometry/configuration, area

and material composition), and operation (hydrodynamics). It is critically determined by the tendency of the membrane to be fouled by feed water components owing to their accumulation on the internal and external structures of the membrane (Visvanathan *et al*., 1989; Belfort *et al*., 1994). This increases the overall resistance to filtration (Belfort *et al*., 1994), thereby commensurately increasing the energy demands. The requirement for cleaning adds to the overall cost, as forces to membrane replacement in cases where cleaning fails to produce adequate flux recovery.

Fouling most commonly takes place external to the membrane, forming a dynamic layer at the membrane surface. As most membrane processes operate in the crossflow mode, fouling through the formation of such a dynamic layer might be expected to reach equilibrium once the adhesive forces between the layer and the membrane substrate are balanced by the shear forces at the layer solution interface. In practice, equilibrium is not always attained, indicating some component of the overall hydraulic resistance to be time dependent.

The flux decline rate generally decreases with time but increases with increasing operating flux or pressure, and the specific flux almost always increases with decreasing trans-membrane pressure (TMP).

1.4.2. Membrane materials and modules

There are a number of different types of membrane materials, modules and associated systems that are utilized by the various classes of membrane filtration. While several different types of membrane modules may be used for any single membrane filtration technology, each class of membrane technology is typically associated with only one type of membrane module in water treatment applications. In general, MF and UF use hollow-fibre membranes and NF and RO use spiral-wound membranes. MCF (Membrane cartridge filtration) systems use flat sheet material configured into a cartridge filtration device. The terms hollow-fibre, spiral-wound, and cartridge refer to the module in which the membrane media is manufactured.

1.4.2.1. Membrane materials

The membrane material refers to the substance from which the membrane itself is made. Normally, the membrane material is manufactured from a synthetic polymer, although other forms, including ceramic and metallic membranes, may be available. Currently, almost all membranes manufactured for drinking water production are made of polymeric material, since they are significantly less expensive than membranes constructed of other materials.

The material properties of the membrane may significantly impact the design and operation of the filtration system. For example, membranes constructed of polymers that react with oxidants commonly used in drinking water treatment should not be used with chlorinated feed water. Mechanical strength is another consideration, since a membrane with greater strength can withstand larger transmembrane pressure (TMP) allowing for greater operational flexibility and the use of higher pressures with pressure-based direct integrity testing.

MF and UF membranes may be constructed from a wide variety of materials, including cellulose acetate (CA), polyvinyledene fluoride (PVDF), polyacrylonitrile (PAN), polypropylene (PP), polysulfone (PS), polyethersulfone (PES), or other polymers. Each of these materials has different properties with respect to surface charge, degree of hydrophobicity, pH and oxidant tolerance, strength, and flexibility. NF and RO membranes are generally manufactured form cellulose acetate or polyamide materials.

1.4.2.2. Membrane modules

Membrane filtration media is usually manufactured as flat sheet stock or as hollow fibres and then configured into one of several different types of membrane modules. A membrane module represents the smallest discrete filtration unit in a membrane system. Module construction typically involves potting or sealing the membrane material into a corresponding assembly, which may incorporate an integral containment structure, such as with hollow-fibre modules. These types of modules are designed for long-term use over the course of a number of years. Spiral-wound modules are also manufactured for long-term use, although the design of membrane filtration systems that utilize spiral-wound

modules requires that the modules be encased in a separate pressure vessel that is independent of the module itself. Alternatively, a module may be configured as a disposable cartridge with a useful life that is typically measured in weeks or months rather than years. Membrane cartridges may either be inserted into pressure vessels that are separate from the module (as with spiral-wound modules) or manufactured within a casing that serves as an integral pressure vessel.

Hollow-Fibre modules

Most hollow-fibre modules used in drinking water treatment applications are manufactured to accommodate porous MF or UF membranes and designed to filter particulate matter. As the name suggests, these modules are comprised of hollow-fibre membranes, which are long and very narrow tubes that may be constructed of any of the various membrane materials. The fibres may be bundled in one of several different arrangements. In one common configuration used by many manufacturers, the fibres are bundled together longitudinally, potted in a resin on both ends, and encased in a pressure vessel that is included as a part of the hollow-fibre module. These modules are typically mounted vertically, although horizontal mounting may also utilized. One alternate configuration is similar to spiral-wound modules in that both are inserted into pressure vessels that are independent of the module itself. These modules (and the associated pressure vessels) are mounted horizontally. Another configuration in which the bundled hollow fibres are mounted vertically and submerged in a basin does not utilize a pressure vessel. A typical commercially available hollow-fibre module may consist of several hundred to over 10,000 fibres. Although specific dimensions vary by manufacturer, approximate ranges for hollow-fibre construction are as follows:

- Outside diameter: 0.5-2.0 mm
- Inside diameter: 0.3-1.0 mm
- Fibre wall thickness: 0.1-0.6 mm
- Fibre length: 1-2 meters

A cross section of a symmetric hollow-fibre is shown in Figure 1.8.

Hollow-fibre membrane modules may operate in either and "inside-out" or "outside-in" mode. In inside-out mode, the feed water enters the fibre lumen (i.e., centre or bore of the fibre) and is filtered radially through the fibre wall. The filtrate is then collected from outside of the fibre. During outside-in operation, the feed water passes from outside the fibre through the fibre wall to the inside, where the filtrate is collected in the lumen. Although inside-out mode utilizes a well-defined feed flow path that is advantageous when operating under a crossflow hydraulic configuration, the membrane is somewhat more subject to plugging as a result of the potential for the lumen to become clogged. The outside-in mode, utilizes a less well-defined flow feed flow path, but increases the available membrane surface area for filtration per fibre and avoids potential problems with clogging of the lumen bore.

Both the inside-out and outside-in operating modes for hollow-fibre modules are illustrated in Figure 1.9. When a hollow-fibre module is operated in an inside-out mode, the pressurized feed water may enter the fibre lumen at one or both ends of the module, with the filtrate exiting in the centre or end(s). In outside- in mode, the feed water typically enters the module in the centre at the ends and is filtered into the fibre lumen, where the filtrate collects before exiting at the end(s). Most hollow- fibre systems operate in "dead-end" or direct filtration mode and are periodically backwashed to remove the accumulated solids.

This module has been widely used for wastewater treatment (Tac-Hyum *et al*., 2003; Fatone *et al*., 2006; Artiga *et al*., 2005a), drinking water treatment and wastewater reclamation (Buttiglieri *et al*., 2005; Arrojo *et al.,* 2005; Melin et al., 2006).

Artiga *et al.* (2005a) used a hollow-fiber ultrafiltration membrane (Zenon ZW-10) with a pore size 0.04 μm and a nominal surface area of 0.9 m^2 for the treatment of two industrial wastewaters (winery and tannery) in a submerged membrane bioreactor. They obtained COD removal percentages around 97% and 86% for winery and tannery wastewater, respectively. Artiga *et al.* (2005b) developed an innovative membrane-assisted hybrid bioreactor for treatment of tannery and fish canning wastewater. This system was composed by a hybrid circulating bed reactor coupled in series to an ultrafiltration hollow fiber module (Zenon, ZW-1). COD removal efficiency was 95%, while up to 97% of ammonia

removal was obtained. Moreover, the membrane filtration unit made it feasible to operate the reactor at high OLR without problems related to either the settling properties of the sludge or the drop in the nitrogen conversion, which usually occurs in other hybrid biological reactors operated at high OLR and NLR.

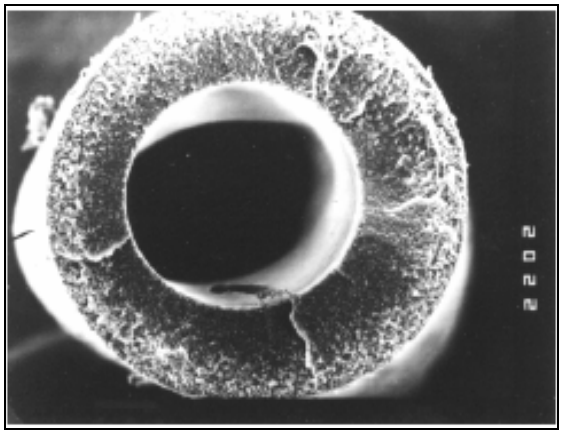


Figure 1.8. Hollow Fibre Cross-Section Photomicrograph. (EPA, 2003)

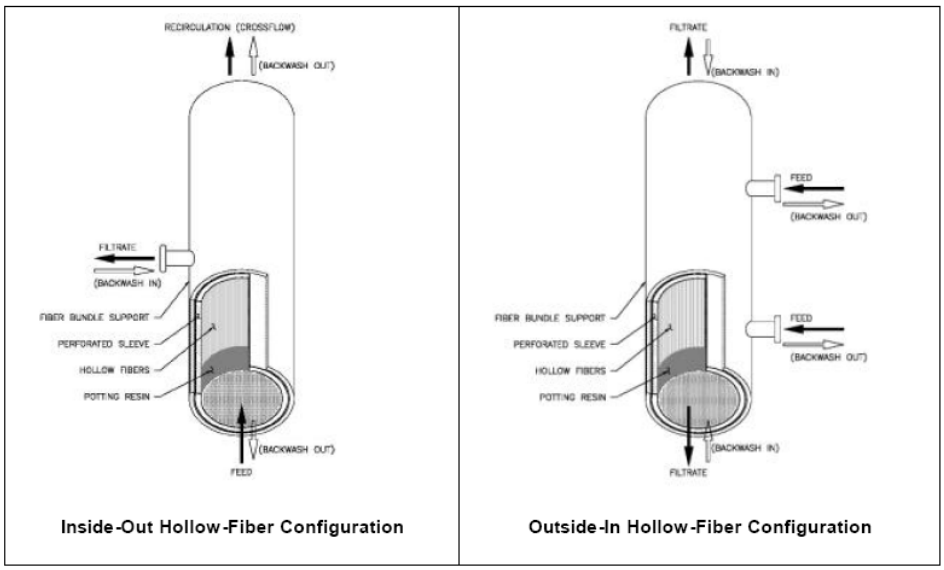


Figure 1.9. Inside-Out and Outside-In modes of operation. (EPA, 2003)

Spiral-Wound Modules

Spiral-wound modules were developed as an efficient configuration for the use of semipermeable membranes to remove dissolved solids, and thus are most often associated with NF/RO processes. The basic unit of a spiral-wound module is a sandwich arrangement of flat membrane sheets called a leaf wound around a central, perforated tube. One leaf consists of two membrane sheets placed back to back and separated by a fabric spacer called a permeate carrier. The layers of the leaf are glued along three edges, while the unglued edge is sealed around the perforated central tube. A single spiral-wound module 8 inches in diameter may contain up to approximately 20 leaves, each separated by a layer of plastic mesh called a spacer that serves as the feed water channel. Feed water enters the spacer channels at the end of the spiral-wound element in a path parallel to the central tube. As the feed water flows across the membrane surface through the spacers, a portion permeates through either of the two surrounding membrane layers and into the permeate carrier, leaving behind any dissolved and particulate contaminants that are rejected by the semi-permeable membrane. The filtered water in the permeate carrier travels spirally inward around the element toward the central collector tube, while the water in the feed spacer that does not permeate through the membrane layer continues to flow across the membrane surface, becoming increasingly concentrated in rejected contaminants. This concentrate stream exits the element parallel to the central tube through the opposite end from which the feed water entered. A diagram of a spiral-wound element is shown in Figure 1.10.

Spiral-wound membranes for drinking water treatment are commercially available in a variety of sizes. Modules that are either 10 or 20 cm in diameter and either 100 or 150 cm long are most common, although other sizes may be used. Some bench- and pilot-scale applications utilize modules that are 6.5 cm in diameter, while modules up to 40 cm in diameter or more may be used in full-scale facilities (EPA, 2003).

These membranes have been used for water desalination and organic removal for water reuse in the food industry (Mavrov *et al.,* 2001), for purification and regeneration of washing solutions from dairy processes (Räsänen *et al.,* 2002) and for volatile organic compound removal (Alvarez *et al*, 2001).

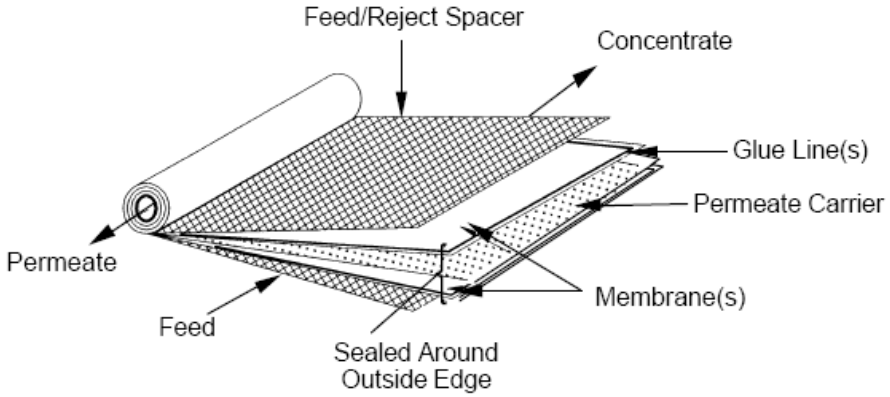


Figure 1.10. Spiral-Wound Membrane module. (EPA, 2003)

Membrane Cartridges

Membrane cartridge filters are manufactured by placing flat sheet membrane media between a feed and filtrate support layer and pleating the assembly to increase the membrane surface area within the cartridge. The pleat pack assembly is then placed around a centre core with a corresponding outer cage and subsequently sealed, via adhesive or thermal means, into its cartridge configuration. End adapters, typically designed with a double o-ring sealing mechanism, are attached to the filter to provide a positive seal with the filter housing. A diagram of membrane cartridge filter is shown in Figure 1.11.

Most membrane cartridge filters are manufactured as disposable components that are inserted into housing. Once the filter fouls to the point at which the maximum transmembrane pressure (TMP) is reached, the cartridge is replaced. Because the cartridges are designed to be disposable, and thus relatively inexpensive to replace, cartridge filtration systems have not historically utilized backwashing or chemical cleaning. However, some systems that feature these processes have recently been introduced. Cartridge filters are available in various sizes and pore sizes, although the device would have to be constructed using a non-fibrous membrane barrier and be capable of filtering particulate matter larger than 1 mm to be considered a membrane filter (EPA, 2003).

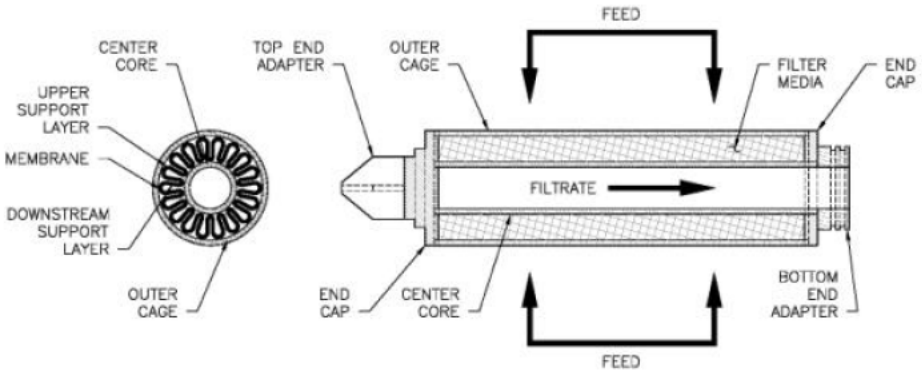


Figure 1.11. Membrane Cartridge filter. (EPA, 2003)

Other Module Configurations

In addition to hollow-fibre and spiral-wound modules, there are several other types of less common configurations that may be used in membrane filtration systems. These configurations include hollow- fine- fibre (HFF), tubular and plate-and-frame type modules (Li *et al*., 2006; Haneda *et al*., 2006).

Semi-permeable HFF membranes were the original hollow-fiber type membranes and were developed for desalting (i.e., RO) applications. With the development of widely used porous hollow- fiber MF and UF membranes for particulate filtration with much larger fiber diameters, the semi-permeable variety gradually became known as hollow-*fine*-fiber membranes. HFF membranes are bundled length-wise and shaped into a "U" arrangement (called a "Utube"), which is potted in a cylindrical pressure vessel.

Tubular membranes are essentially a larger, more rigid version of hollow-fibre membranes (Figure 1.12). With diameters as large as 2-5 cms, the tubes are not prone to clogging and the membrane material (i.e., the tube wall) is comparatively easy to clean. However, the large tubes also result in a very inefficient amount of membrane surface area per unit volume in the pressure vessel. Both porous (for MF/UF) and semi-permeable (for NF/RO) membranes have been manufactured in tubular configurations. Ceramics have been considered as non-traditional material for tubular MF/UF membranes, although there are

currently no commercially promoted ceramic MF/UF systems for drinking water applications.

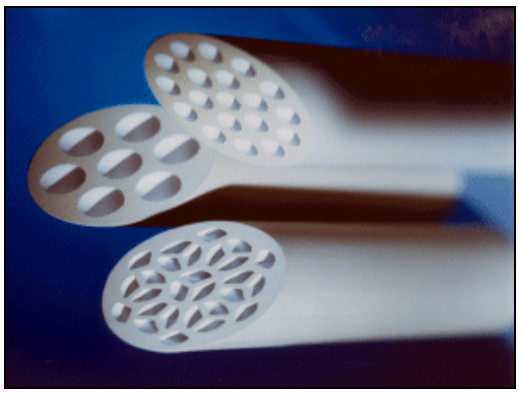


Figure 1.12. Ceramic tubular membranes. (EPA, 2003)

A plate and frame configuration, one of the earliest membrane modules developed, is simply a series of flat sheet membranes separated by alternating filtrate spacers and feed/concentrate spacers. Because of the very low surface area to volume ratio, the plate-and-frame configuration is considered inefficient and is therefore seldom used in drinking water applications.

To sum up, membrane technology are very compact wastewater treatment systems in which a better effluent quality is produced and offer an alternative to conventional process for the treatment of wastewaters. The use of membranes could be very attractive when either wastewater reclamation is one of the objectives of the treatment or a more stringent effluent quality is required.

1.5. References

Alphenaar P.A., Visser A. and Lettinga G. (1993). The effect of liquid upflow velocity and hydraulic retention time on *granulation in UASB reactors treating wastewater with a high-sulphate content. Bioresource Technology*, **43**, 249-258.

Alvarez F.R., Vane L.M. and Lynnann H. (2001). Demonstration of pilot-scale pervaporation systems for volatile organic compound removal from a surfactant enhanced aquifer remediation fluid I: spiral wound membrane modules. *Environmental Progress*, **20** (1), 53-63.

Alves M., Cavaleiro A.J., Ferreira E.C., Amaral A.L., Mota M., da Motta M., Vivier H. and Pons M.N. (2000). Characterization by image analysis of anaerobic sludge under shock conditions. *Water Science and Technology*, **41**, 207-214.

Anderson I.C. and Levine J.S. (1986). Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers and nitrate respires. *Applied and Environmental Microbiology*, **51** (5), 938-945.

Anderson A.J. and Dawes E.A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbioogy Reviews*, **54**, 450-472.

Andreottola G., Folodori F. and Ragazzi M. (2001). On-line control for a SBR system for nitrogen removal from industrial wastewater. *Water Science and Technology*, **43** (3), 93-100.

Arrojo B., Mosquera-Corral A., Garrido J.M. and Méndez R. (2004) Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research*, **38**, 3389 – 3399.

Arrojo B**.**, Mosquera-Corral A., Garrido J.M., Méndez R., Ficara E. and Malpei F. (2005). Membrane coupled to a Sequencing Batch Reactor for water reuse and removal of coliform bacteria. *Desalination***, 179**, 109-116**.**

Artiga P., Ficara E., Malpei F., Garrido J.M. and Méndez R. (2005a). Treatment of two industrial wastewaters in a submerged membrane bioreactor. *Desalination*, **179**, 161-169.

Artiga P., Oyanedel V., Garrido J.M. and Méndez R. (2005b). An innovative biofilm-suspended biomass hybrid membrane bioreactor for wastewater treatment. *Desalination*, **179**, 171-179.

Banas J., Plaza E., Styka W. and Trela J. (1999). SBR technology used for advanced combined municipal and tannery wastewater treatment with high receiving water standards. *Water Science and Technology*, **40** (4-5), 451-8.

Belfort, G., R. H. Davis, and A. Zydney. (1994). The Behavior of Suspensions and Macromolecular Solutions in Crossflow Microfiltration. *Journal Membrane Science*, **96**, 1-58.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P.A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research* **33** (10), 2283-2290.

Beun J. J., van Loosdrecht M. C. M. and Heijnen J. J. (2000). Aerobic granulation. *Water Science and Technology*, **41**(4-5), 41-48.

Beun J.J., van Loosdrecht M.C.M. and Heijnen J.J. (2001) N-removal in a granular sludge sequencing batch airlift reactor. *Biotechnology and Bioengineering*, **75**(1), 82-92.

Beun J.J., van Loosdrecht M.C.M. and Heijnen J.J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research*, **36**, 702-712.

Bock E., Koops H., Ahlers B. and Harms H. (1992). Oxidation of inorganic compounds as energy source. In A.Balows, H.G. Trüper, M. Dworkin, W. Harder and K.H. Schleifer, *The Prokaryotes: A Hand-book on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications,* eds, 414-430. Springer, New York

Bosier P. and Verstraete W. (1996). Triggers for microbial aggregation in activated sludge? *Applied Microbiology Biotechnology*, **45**, 1-6.

Broda E. (1977). Two kinds of lithotrophs missing in nature. *Zeitschrift für allgemeine Microbiología*, **17**, 491-493.

Buisson H., Cote P., Praderie M. and Paillard H. (1998). The use of immersed membranes for upgrading wastewater treatment plants. *Water Science and Technology* **37** (9), 89-95.

Butler M.D., Stephenson T., Stokes L. And Stuetz R.M. (2005). Dinitrogen oxide detection for nitrification failure early warning systems. *Water Science and Technology*, **52**(8), 249-256.

Buttiglieri G., Malpei F., Daverio E. Melchiori M., Nieman H. and Ligthart J. (2005). Denitrification of drinking water sources by advanced biological treatment using a membrane bioreactor. *Desalination*, **178**, 211-218.

Byron D. (1987). Polymer synthesis by micro-organisms: technology and economics. *Trends in Biotechnology*, **5**, 246-250.

Chiemchaisri, C.; Wong, Y. K.; Urase, T.; Yamamoto, K. (1992). Organic stabilization and nitrogen removal in membrane separation bioreactor for domestic wastewater treatment. *Water Science and Technology*, **25** (10), 231-40.

Chiemchaisri C. and Yamamoto K. (1994). Performance of membrane separation bioreactor at various temperature for domestic wastewater treatment. *Journal of Membrane Science*, **87**, 11-129.

Chisti Y. (1999). Shear sensitivity. In: Flickinger MC., Drew SW., editors. Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol 5. New York: Wiley; 2379-406.

Choi J. and Lee S.Y. (1997). Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. *Bioprocess Engineering*, **17**, 335-342.

Côté P., Buisson H., Pound Ch. and Arakaki G. (1997). Inmersed membrane activated sludge for the reuse of municipal wastewater. *Desalination*, **113**, 189-196.

Countil Directive of 8 December 1975 (1976). Concerning the Quality of Bathing Water, Official. J. Eur. Commun., L31.

Countil Directive of 21 May 1991 (1991). Concerning Urban Waste Water Treatment, Official J.Eur. Commun., L135.

Dangcong P., Bernet N., Delgenes J.-P. y Moletta R. (1999). Aerobic granular sludge-a case report. *Water Research*, **33**(3), 890-893.

Dapena-Mora A., Campos J.L., Mosquera-Corral A., Jetten M.S.M. and Méndez R. (2004a). Stability of the Anammox Process in a gas-lift reactor and a SBR. *Journal of Biotechnology*, **110** (2), 159-170.

Dapena-Mora A., Van Hulle S., Campos J.L., Méndez R., Vanrolleghem P.A. y Jetten M.S.M. (2004b). Enrichment of Anammox biomass from municipal activated sludge: experimental and modelling results. *Journal of Chemical Technology and Biotechnology*, **79**, 1421-1428.

Dapena-Mora A., Arrojo B., Campos J.L., Mosquera-Corral A. y Méndez R. (2004c). Improvement of the settling properties of Anammox sludge in an SBR. *Journal of Chemical Technology and Biotechnology*, **79**, 1417-1420.

Dapena-Mora A., Campos J.L., Mosquera-Corral A. and Méndez R. (2006a). Anammox process for nitrogen removal from anaerobically digested fish canning effluents. *Water Science and Technology*, **53 (**12), 265-274.

Dapena-Mora A., Fernández I., Campos J.L., Mosquera-Corral A.,Méndez R and Jetten M.S.M. (2006b). Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology* (*In press*).

Dawes E.A. and Senio P.J. (1973). The role and regulation of energy reserve polymers in microorganisms. *Advances in Microbial Physiology*, **10**, 135-266.

de Bruin L.M.M., de Kreuk M.K., van der Roest H.F.R., Uijterlinde C. and van Loosdrecht M.C.M. (2004). Aerobic granular sludge technology: and alternative to activated sludge. *Water Science and Technology*, **49** (11-12),1-7.

de Bruin L.M.M., van der Roest H.F., de Kreuk M.K. and van Loosdrecht M.C.M. (2005). Promising results pilot research aerobic granular sludge technology at WWTP Ede. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing*. Munich, (135-142).

de Kreuk M.K., McSwain B.S., Bathe S., Tay S.T.L., Schwarzenbeck and Wilderer P.A. (2005). Discussion outcomes. Ede. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing*. Munich, (165-169).

de Kreuk M.K. and van Loosdrecht M.C.M. (2006). Formation of aerobic granules with domestic sewage. *Journal of Environmental Engineering*, **132** (6), 694-697.

Doi, (1990), Microbial polyesters, New York, VCH Publishers. 156.

Doi Y., Kawaguchi Y., Koyama N., Nakamura S., Hiramitsu M., Yoshida Y. and Kimura H. (1992). Synthesis and degradation of polyhydroxyalkanoates in Alcaligenes eutrophus. *FEMS Microbiology Reviews*, **103**, 103-108.

Egli K., Fanger U., Alvarez P.J.J., Siegrist H., van der Meer J.R. and Zehnder A.J.B. (2001). Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. *Archives of Microbiology*, **175**, 198-207.

EPA. (1999). Wastewater technology fact sheet sequencing batch reactor. Office of Water, United States Environmental Protection Agency, Washington DC.

EPA(2003). Membrane Filtration Guidance Manual. United States Environmental Protection Agency.

Etterer T. and Wilderer P.A. (2001). Generation and properties of aerobic granular sludge. *Water Science and Technology* **43** (3), 19-26.

Fang H.H.P. and Chui H.K. (1993). Maximum COD loading capacity in UASB reactors at 37ºC. *Journal Environmental Engineering*, **119**, 103-119.

Fatone F., Battistoni P., Pavan P. and Cecchi F. (2006). Application of a membrane bioreactor for the treatment of low loaded domestic wastewater for water re-use. *Water Science and Technology*, **53** (9), 111-121.

Gander M., Jefferson B. and Judd S. (2000). Aerobic MBRs for domestic wastewater treatment: a review with cost considerations. *Separation and Purification Technology* **18**, 119-130.

Garrido, J.M., Moreno, J., Méndez-Pampín, R. and Lema, J.M. (1998). Nitrous oxide production under toxic conditions in a denitrifying. *Water Research*, **32**, 2550-2552.

Gejlbjerg B., Frette L. and Westermann P. (1998). Dynamics of N_2O production from activated sludge. *Water Research*, **32** (7), 2113-2121.

Goreau T.J., Kaplan W.A., Wofsy S.C., McElroy M.B., Valois F.W. and Wattson S.W. (1980). Production of NO_x^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Applied and Environmental Microbiology*, **40** (3), 526-532.

Guiot S.R., Pauss A. and Costerton J.W. (1992). A structured model of the anaerobic granule consortion. *Water Science and Technology*, **25** (7), 1-10.

Haywood G.W., Anderson A.J. and Dawes E.A. (1989). The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by Alcaligenes eutrophus. *FEMS Microbiology Letters*, **57**, 1-6.

Haneda R.N., Ikegami R., Formulan C.A., Purqueiro B.M., Longo E., Fones S.R. (2006). Microfiltration with chemistry treating of comercial membranes and microporous tubes for retention of bacteria *E.Coli* on processing of wastewater of dairy products. *Desalination*, **200** (1-3), 313-315.

Heijnen J.J., Mulder A., Weltevrede R., Holds P.H. and Van Leeuwen H.L.J.M. (1990). Large-scale anaerobic/aerobic treatment of complex industrial wastewater using immobilized biomass in fluidized bed and air-lift suspension reactors. *Chemical Engineering Technology*, **13**, 202-208.

Helmer C., Tromm C., Hippen A., Rosenwickel K.H., Seyfried C.F. y Kunst S. (2001). Single stage biological nitrogen removal by nitritation and anerobic ammonium oxidation in biofilm systems. *Water Science and Technology*, **43**, 311-320.

Henze M. (1991). Capabilities of biological nitrogen removal processes from wastewater. *Water Science and Technology*, **23**, 669-679.

Hu L., Wang J., Wen X. and Qian Y. (2005). The formation and characteristics of aerobic granules in sequencing batch reactor (SBR) by seeding anaerobic granules. *Process Biochemistry*, **40**, 5-11.

Hulshoff P.L.W., Heijnekamp K. and Lettinga G. (1988). The selection pressure as a driving force behing the granulation of anaerobic sludge. In: Lettinga G., Zehnder A.J.B., Grotenhuis J.T.C., Hulshoff Pol L.W., editors. Granular anaerobic sludge: microbiology and technology. Wageningen: GASMAT, Lunteren; 153-161.

Hulshoff P.L.W. (1989). The phenomenon of granulation of anaerobic sludge. PhD thesis, Wageningen Agricultural University, The Netherlands.

Humphreys P.N. and Banks C.J. (1995). The use of sequencing batch activated sludge reactors to determine nitrogen balances and optimum periods of pre-aeration denitrification. *Environmental Technology*, **16**, 549-558.

Iaconi Di C., Ramadori R., López A. and Passino R. (2006). Influence of hydrodynamic shear force on properties of granular biomass in a sequencing batch biofilter reactor. *Biochemical Engineering Journal*, **30**, 152-157.

Irvine R.L., Ketchum L.H., Breyfogle R. and Barth E.F. (1983). Municipal application of sequencing batch treatment. *Journal Water Pollution Control Federation*, **55**, 484-488.

Irvine R.L., Wilderer P.A. and Flemming H.C. (1997). Controlled unsteady state processes and technologies-an overview. *Water Science and Technology*, **35**, 1-10.

Jetten M.S.M., Horn S.J. y van Loosdrecht M.C.M. (1997). Towards a more sustainable wastewater treatment system. *Water Science and Technology*, **35**(9), 171-179.

Jetten M.S.M. (1999). New pathways for ammonia conversion in soil and aquatic systems. *Plant & Soil*, **230**, 9-19.

Jiang H.L., Tay J-H and Tay S.T.L. (2002). Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol.. *Letters in Applied Microbiology*, **63**, 602-608.

Jiang H.L., Tay J.H., Liu Y. and Tay S.T.L. (2003). Ca^{2+} augmentation for enhancement of aerobically grown microbial granules in sludge blanket reactors. *Biotechnology Letters*, **25**, 95-99.

Judd, S. J.and Till, S. W. (2000). Bacterial rejection in crossflow microfiltration of sewage. *Desalination*, **127** (3), 251-260.

Kang I.J., Lee C.H. and Kim K.J. (2003). Characteristics of microfiltration membranes in a membrane coupled sequencing batch reactor system. *Water Research* **37**, 1192-1197.

Ketchum Jr. LH. (1997). Design and physical features of sequencing batch reactors. *Water Science and Technology,* **35** (1), 11-8.

Kishida N., Kim J., Tsuneda S. and Sudo R. (2006). Anaerobic/oxic/anoxic granular sludge process as an effective nutrient removal process utilizing denitrying polyphosphate-accumulating organisms. *Water Research*, **40**, 2303-2310.

Kishino H., Ishida H., Iwabu H. and Nakano I. (1996). Domestic wastewater reuse using a submerged membrane bioreactor. Desalination 106 115-119.

Kolega, M.; Grohmann, G. S.; Chiew, R. F.; Day, A. W. (1991). Disinfection and clarification of treated sewage by advanced microfiltration. Water Science and Technology, **23** (7-9), 1609-18.

Krampe J. and Krauth K. (2000). Sequencing batch reactor with submerged hollow fibre membranes for the biomass separation. In: Proceedings of 2nd international symposium on sequencing batch reactor technology. Vol 2., 109-15.

Kuypers M.M.M., Sliekers A.O., Lavik G., Schmid M., Jorgensen B.B., Kuenen J.G., Sinninghe Damste J.S., Strous M. and Jetten M.S.M. (2003). Anaerobic ammomium oxidation by Anammox bacteria in the Black Sea. *Nature*, **422**, 608-611.

Kwok W.K., Picioreanu C., Ong S.L., van Loosdrecht M.C. M., Ng W.j. and Heijnen J.J. (1998). Influence of biomass production and detachment forces on biofilm structures in a biofilm airlif suspension reactor. *Biotechnology and Bioengineering*, **58**, 400-407.

Lee S.Y. (1996). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends in Biotechnology*, **14**, 431-438.

Lettinga G., van Velsen A.F.M., Hobma S.W., de Zeeuw W. and Klapwijk A. (1980). Use of the Upflow Sludge Blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering*, **22**, 699-734.

Li Y.S., Yan L.X., Chai Bao H. and Liu J. (2006). Treatment of oily wastewater by organic-inorganic composite tubular ultrafiltration (UF) membranes. *Desalination*, **196** (1-3).

Lin Y.M., Liu Y. and Tay J.H. (2003). Development and characteristics of phosphorous-accumulating granules in sequencing batch reactor. *Applied Microbiology Biotechnology*, **62**, 430-435.

Liu Y., Xu H.L., Yang S.F. and Tay J.H. (2003). Mechanisms and models for anaerobic in upflow anaerobic sludge blanket reactor. *Water Research,* **37**, 661-673.

Liu Y., Yang S.F., Tay J-H, Liu Q.S., Qin L. and Li Y. (2004a). Cell hydrophobicity is a triggering force of biogranulation. *Enzyme Microbiology Technology*, **34**, 371-379.

Liu Y. and Liu, Q.-S. (2006). Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. *Biotechnology Advances*, **24**(1), 115-127.

Liu Y-Q. and Tay J-H. (2006). Variable aeration in sequencing batch reactor with aerobic granular sludge. *Journal of Biotechnology*, **124**, 338-346.

Madaeni, S. S.; Fane, A. G.; Grohmann, G. S. (1995). Virus removal from water and wastewater using membranes. *Journal of Membrane Science,* **102** (1-3), 65-75.

Martins A. M. P., Heijnen J. J., van Loosdrecht M. C. M. (2003). Effect of feeding pattern and storage on the sludge settleability under aerobic conditions. *Water Research*, *37*(11), 2555-2570.

Mavrow V., Chmiel H. and Bélières (2001). Spent process water desalination and organic removal by membranes for water reuse in the food industry. *Desalination*, **138**, 65-74.

Mayhew M. and Stephenson T. (1997). Low biomass yield activated sludge: a review. *Environmental Technology*, **18**, 883.

Melin T., Jefferson B., Bixio D., Thoeye C., De Wilde W., De koning J., van der Graaf J. And Wontgens T. (2006). Membrane bioreactor technology for wastewater treatment and reuse. *Desalination*, **178**, 271-282.

Mohan S.B., Schmid M., Jetten M. and Cole J. (2004). Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. *Fems Microbiology Ecology* **49** (3), 433-443.

Morgenroth E., Sherden T., Van Loosdrecht M.C.M., Heijnen J.J. and Wilderer P.A. (1997). Aerobic granular sludge in a sequencing batch reactor. *Water Research*, **31** (12), 3191-3194.

Mosquera-Corral A., Vázquez-Padín J.R., Arrojo B., Campos J.L. and Méndez R. (2005a). Nitrifying granular sludge in a sequencing batch reactor. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing.* Munich, (63-70).

Mosquera-Corral A., de Kreuk M.K., Heijnen J.J. and van Loosdrecht M.C.M. (2005b). Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. *Water Research*, **39** (12), 2676-2686.

Mourato D., Behmann H. and McGinn G. (1996). The Zenogem process for municipal sewage treatment plant upgrades. *In proceedings of IWSA-EDS Workshop on Membranes in Drinking Water Production*, L'Aquila, Italy.

Moy B.Y.P., Tay J.H., Toh S.K. Liu Y. and Tay S.T.L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Letters in Applied Microbiology*, **34,** 407-412.

Mulder A., van de Graaf A.A., Robertson L.A. y Kuenen J.G. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology*, **16**, 177-184.

Mulder A. (1992). Anoxic Ammonium Oxidation US patent 427849(5078884). Patente en USA.

Ohashi A. and Harada H. (1994). Adhesion strength of biofilms developed in an attached-growth reactor. *Water Science and Technology*, **29**, 10.

Painter H.A. and Loveless J.E. (1983). Effect of temperature and pH values on the growth rate constants of nitrifying bacteria in the activated sludge process. *Water Research*, **17**, 237-248.

Pavelj N., Hvala N., Kocijan J., Ro M., Ubelj M., Mui G. and Strmnik S. (2001). Experimental design of an optimal phase duration control strategy used in batch biological wastewater treatment. *ISA Transactions*, **40** (1), 41-56.

Peng D., Bernet N., Delgenes J.P. and Moleta R. (1999). Aerobic granular sludge-a case report. *Water Research*, **33,** 890-893.

Picioreanu C., Van Loodsdrecht M.C.M. and Heijnen J.J. (2000). Effect of diffusive and convective substrate transport on biofilm structure formation: a two-dimensional modelling study. *Biotechnology and Bioengineering*, **69**, 504-515.

Pochana K. and Keller J. (1999). Study of factors affecting simultaneous nitrification and denitrification (SND). *Water Science and Technology*, **39** (6), 61-8.

Poth M. and Focht D. (1985). ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. *Applied and Environmental Microbiology*, **49** (5), 1134-1141.

Qin L., Tay J-H and Liu Y. (2004a). Selection pressure is a driving force of aerobic granulation in sequencing batch reactors. *Process Biochemistry*, **39**, 579-584.

Qin L. Liu Y. and Tay J-H (2004b). Effect of settling time on aerobic granulation in sequencing batch reactor. *Biochemistry Engineering Journal*, **21**, 47-52.

Ramadori R., Iaconi Di C., López A. and Passino R. (2006). An innovative technology based on aerobic granular biomass for treating municipal and/or industrial wastewater with low environmental impact. *Water Science and Technology*, **53** (12), 321-329.

Räsänen E., Nyström M., Sahlstein J. and Tossavainen O. (2002). Purification and regeneration of diluted caustic and acid washing solutions by membrane filtration. *Desalination*, **149**, 185-190.

Schleypen P., Michel I. and Siewert H.E. (1997). Sequencing batch reactors with continuous inflow for small communities in rural areas in Bavaria. *Water Science and Technology*, **35** (1), 269-276.

Schmidt J.E. and Ahring B.K. (1996). Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors.*Biotechnology and Bioengineering*, **49**, 229-246.

Schmid M., Twachtmann U., Klein M., Strous M., Juretschko S., Jetten M., Metzger J, Schleifer K.H. y Wagner M. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Systematic and Applied Microbiology*, **23**, 93-106.

Schmidt M., Schmitz-Esser S., Jetten M. and Wagner M. (2001). 16S-23rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental. Microbiology,* **3** (7), 450-9.

Schmidt I., Hermelink C., van de Pas-Schoonen K., Strous M., Camp H.J., Kuenen J.G.and Jetten M.S.M. (2002). Anaerobic ammonia oxidation in the presence of nitrogen oxides (NOx) by two different lithotrophs. *Applied Enviromental Microbiology,* **68** (11), 5351-5357.

Schmid M., Walsh K., Webb R. Rijpstra WIC, van de Pas-Schoonen K.T., Verbruggen M.J., Hill T., Moffett B., Fuerst J., Schouten S., Sinninghe Damste JS, Harris J., Shaw P.ñ, Jetten MSM and Strous M. (2003).

Candidatus "Scalindua brodae", sp nov., *Candidatus* "Scanlindua wagneri", sp nov., two species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology*, **26**, 529-538.

Schwarzenbeck N., Erley R. and Wilderer P.A. (2004). Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter. *Water Science and Technology*, **49** (11-12), 41-46.

Schwarzenbeck N. and Wilderer P.A. (2005). Treatment of food industry effluents in a granular sludge SBR. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing*. Munich, (95-102).

Schulthess R., Wild D. and Gujer W. (1994). Nitric and nitrous oxide from denitrifying activated sludge at low oxygen concentration. *Water Science and Technology*, **30** (6), 123-132.

Shaw L.J., Nicol G.W., Smith Z., Fear J., Prosser J. and Baggs E.M. (2006). Nitrosospira spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Applied and Environmental Microbiology*, **8** (2), 214-222.

Shin H. S., Lim K.H. and Park H. S. (1992). Effect of shear stress on granulation in oxygen aerobic upflow sludge reactors. *Water Science and Technology*, **26**, 601-605.

Steinbuchel A. (1996). PHB and other polyhydroxyalkanoic acids. In: HJ Rehm, G.Reed. Biotechnology. Products of primary metabolism. Weinheim: Verlag Chemie. 405-464.

Strenstrom M.K. and Poduska R.A. (1980). The effect of dissolved oxygen concentration on nitrification. *Water Research*, **14**, 643-649.

Strous M., van Gerven E., Kuenen J.G. y Jetten M. (1997a). Ammonium removal from concentrated waste streams with the Anaerobic Ammonium Oxidation (anammox) process in different reactor configurations. *Water Research,* **31**, 1955-1962.

Strous M., van Gerven E., Kuenen J.G. y Jetten M. (1997b). Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (anammox) sludge. *Applied and Environmental Microbiology*, **63**, 2446-2448.

Strous M., Heijnen J.J., Kuenen J.G. y Jetten M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology,* **50**, 589-596.

Strous M., Kuenen J.G. y Jetten M. (1999). Key physiological parameters of anaerobic ammonium oxidation. *Applied Microbiology and Biotechnology,* **65**, 3248-3250.

Strous M., Kuenen J.G., Fuerest J.A., Wagner M. and Jetten M.S.M. (2002). The Anammox case- A new experimental manifesto for microbiological eco-physiology. *Antonie van Leuwenhoek.* **81**, 693-702.

Stüven R. and Bock E. (2001). Nitrification and denitrification as a source for NO and NO_2 production in high-strength wastewater. *Water Research,* **35** (8), 1905-1914.

Suthersand S. and Ganczarczyk J.J. (1986). Inhibition of nitrite oxidation during nitrification. Some observations. *Water Research Journal*, **21** (2), 257-266.

Sutka R.L., Ostrom N.E., Ostrom P.H., Breznak J.A. , Gandhi H., Pitt A.J. and Li F. (2006). Distinguishing nitrous oxide production from nitrification and denitrification on the basis of isotopomer abundances. *Applied and Environmental Microbiology*, **72**(1), 638-644.

Tac-Hyun B., Sung-soo H. Tac-Mon T. (2003). Membrane sequencing batch reactor system for the treatment of dairy industry wastewater. *Process Biochemistry*, **39**, 221-231.

Tay J.-H., Liu Q.-S. and Liu Y. (2001a). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters of Applied Microbiology*, **33**, 222–226.

Tay J.-H., Liu Q.-S. and Liu Y. (2001b). The effects of shear force on the formation, structure and metabolism of aerobic granules. *Applied Microbiology and Biotechnology*, **57**, 227-233.

Tay J-H, Liu Q-S y Liu Y. (2001c). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *Journal of Applied Microbiology,* **91**, 168-175.

Tay J.-H., Liu Q.-S. y Liu Y. (2002a). Aerobic granulation in sequential sludge blanket reactor. *Water Science and Technology*, **46**(4-5), 13-18.

Tay J-H, Liu Q-S and Liu Y. (2002b). Hydraulic selection pressure-induced nitrifying granulation in sequencing batch reactors. *Applied Microbiology and Biotechnology,* **59**, 332-337.

Tay J-H., Pand S., He Y.X. and Tay S.T.L. (2003a). Effect of organic loading rate on aerobic granulation: Part II. Characteristics of aerobic granules. *Journal Environmental Engineering,* **130** (10), 1102-1109.

Tay J-H, Liu Q. S. and Liu Y. (2003b). Shear force influences the structure of aerobic granules cultivated in sequencing batch reactor. *5th International Conference on biofilms systems*, 14-19 September, Cape Town, South Africa.

Tay J.-H., Liu Q.-S. and Liu Y. (2004). The effect of upflow air velocity on the structure of aerobic granules cultivated in a sequencing batch reactor. *Water Science and Technology*, **49**, 11-12, 35-40.

Tay J.H., Liu Q.-S., Liu Y., Show K.-Y., Ivanov V. and Tay S.T.-L. (2005). A comparative study of aerobic granulation in pilot- and labratory scale SBRs. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing*. Munich, (125-133).

Tijhuis L., van Loosdrecht M.C.M. and Heijenen J.J.(1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioeng*ineering, **44**, 595-608.

Tijhuis L., Hijman B., van Loosdrecht M.C.M. and Heijnen J.J. (1995). Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. *Applied. Microbiology and Biotechnology,* **45**, 7-17.

Tortoso A.C. and Hutchinson, G.L. (1990). Contributions of autotrophic and heterotrophic nitrifiers to soil NO and N_2O emissions. *Applied and Environmental Microbiology*, **56** (8), 1440-1448.

Tsuneda S., Nagano T., Hoshino T., Ejiri Y., Noda N. and Hirata A. (2003). Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Research*, **37**, 4965-4973.

Tsuneda S., Ejiri Y., Ogiwara M., Nagano T. and Hirata A. (2005). Characteristics and applicability of nitrifying granules produced in an anaerobic upflow fluidized bed reactor. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing.* Munich, (15-24).

Tsuneda S., Ogiwara M., Ejiri Y. and Hirata A. (2006). High-rate nitrification using aerobic granular sludge. *Water Science and Technology*, **53** (3), 147-154.

Ueda T., Hata K. and Kikuola Y. (1996). Treatment of domestic sewage from rural settlements by a membrane bioreactor. *Water Science Technology,* **34** (9), 189-196.

Ueda T., Hata K., Kikuoka Y. and Seino O. (1997). Effects of aeration on suction pressure in a submerged membrane bioreactor. *Water Research*, **31** (3), 489-494.

Ueda T. and Hata K. (1999). Domestic wastewater treatment by a submerged membrane bioreactor with gravitational filtration. *Water Research,* **33** (12), 2888-2892.

van Benthum W.A.J., Van Loosdrecht M.C.M. and Heijnen J.J. (1997). Control of heterotrophic layer formation on nitrifying biofilms in a biofilm airlift suspension reactor. *Biotechnology and Bioengineering*, **53**, 397-405.

van Benthum W.A.J., Garrido J.M., Mathijsen J.P.M., Sunde J., Van Loosdrecht M.C.M. and Heijnen J.J. (1998). Nitrogen removal in an intermittently aerated biofilm airlift reactor. *Journal Environmental Engineering*, **124**, 239-248.

van de Graaf A.A., Mulder A., de Bruijn P., Jetten M.S.M., Robertson L.A. y Kuenen J.G. (1995). Anaerobic oxidation of ammonium is a biologically mediated process. *Applied and Environmental Microbiology,* **61**, 1246-1251.

van de Graaf A.A., de Bruijn P., Robertson L.A., Jetten M.S.M. y Kuenen J.G. (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology (UK),* **142**, 2187-2196.

van Dongen U., Jetten M.S.M. and Van Loosdrecht M.C.M. (2001). The SHARON-ANAMMOX process for treatment of ammonium rich wastewater. *Water Science and Technology*, **44**(1), 153-160.

van Lier J.B., van der Zee F.P., Tan N.C.G., Rebac S. and Kleerebezem R. (2001). Advances in high-rate anaerobic treatment: staging of reactor systems. *Water Science and Technology*, **44** (8), 15-25.

van Loosdrecht M.C.M., Eikelboom D., Gjaltema A., Mulder A., Tijhuis L. And Heijnen J.J. (1995). Biofilm structures. *Water Science and Technology*, **32**, 35-43.

van Loosdrecht M.C.M., Pot M.A. and Heijnen J.J. (1997). Importance of bacterial storage polymers in bioprocesses. *Water Science and Technology*, **35**, 41-47.

van Wegen R.J., Ling Y., and Middelberg A.P.J. (1998). Industrial production of polyhydroxyalkanoates using *Escherichia coli*: An economic analysis. *Trans IChemE*., **76**, 417-426.

Visvanathan, C. and Ben Aim, R. (1989). Studies on colloidal membrane fouling mechanisms in crossflow microfiltration. *Journal of Membrane Science*, **45** (1-2), 3-15.

Wang Q., Du G. and Chen J. (2004). Aerobic granular sludge cultivated under the selective pressure as a driving force. *Process Biochemistry*, **39**, 557-563.

Wang F., Liu Y.-H., Yang F.L., Zhang X.W. and Zhang H.M. (2005). Study on the stability of aerobic granules in a SBAR- effect of the superficial upflow air velocity and carbon source. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing*. Munich, (35-42).

Wilderer P.A., Irvine R.L. and Goronszy M.C. Sequencing batch reactor technology. IWA publishing, London, UK (2001).

Yamamoto K., Hiasa M., Mahmood T. and Matsuo T. (1989). Direct solid-liquid separation using hollow fiber membranes in an activated sludge aeration tank. *Water Science and Technology*, **21** (1), 43-54.

Yang S.F., Liu Y. and Tay J.H. (2003a). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *Journal Biotechnology*, **106**, 77-86.

Yang S.F., Tay J-H and Liu Y. (2003b). Effect of substrate N/COD ratio on the formation of aerobic granules. *Journal Environmental Engineering,* **131** (1), 86-92.

Yoon S-H. Kim H-S and Yeom I.K. (2004). The optimum operational condition of membrane bioreactor (MBR): cost estimation of aeration and sludge treatment. *Water Research*, **38**, 37-46.

Zevenhuizen LPTM. and Ebbink A.G. (1974). Interrelations between glycogen, poly-β-hydroxybutiric acid and lipids during accumulation and subsequent utilization in a Pseudomona. *Antonie van Leeuwenhoek*, **40**, 103-120.

Zhou J.Q. (2004). Contribution of cell starvation to aerobic granulation. MSc thesis, Nanyang Technological University, Singapore.

Chapter 2

Materials and Methods

Summary

In this chapter, the analytical methods used in this work are described. It comprises the conventional parameters used for wastewater (organic matter, nitrogen compounds, pH, dissolved oxygen, solids and carbon compounds concentrations) and the biomass characterisation. The biomass was characterised by means of parameters such as granules density, volumetric sludge index and techniques such as digital image analysis, electronic microscopy and stereomicroscope.

Identification of the different populations present in the biomass samples was researched by Fluorescent In Situ Hybridisation (FISH).

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

2.1. Liquid phase

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

2.1.1. Chemical Oxygen Demand (COD)

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

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

Reagents preparation

a. Standard potassium dichromate digestion solution: 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ are dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 are added. The solution is cooled to room temperature and, finally, diluted to 1000 mL.

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

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

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

e. Standard ferrous ammonium sulphate titrant (FAS) 0.035 N: 13.72 g of $Fe(NH)_4(SO)_2 \cdot 6H_2O$ are dissolved in distilled water. Then, 20 ml of concentrated H_2SO_4 are added and, finally, the solution is cooled and diluted to 1000 mL

Determination procedure

This procedure is applicable to samples with COD concentrations between 90-900 mg/L. Place 2.5 mL of sample in 10-mL Pirex tubes. Add 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). A blank sample using distilled water is prepared in the same way. This blank acts as "reference", representing the COD of the distilled water. After being sealed with Teflon and tightly capped, the tubes are finally mixed completely and placed in the block digester (HACH 16500-100) preheated to 150ºC. The duration of the digestion period is 2 h.

After digestion, the tubes are cooled to room temperature. Then, the content of the tubes is transferred to a beaker and, once added 1-2 drops of ferroin indicator, the solution is titrated under rapid stirring with standard FAS. The FAS solution is standardised daily as follows: Put 5 mL of distilled water into a small beaker. Add 3.5 mL of sulphuric acid reagent. Cool to room temperature and add 5 mL of standard potassium dichromate solution (0.05 N). Add 1-2 drops of ferroin indicator and titrate with FAS titrant. The end-point is a sharp colour change from blue-green to reddish brown. Molarity of FAS solution is calculated with the following equation:

$$M_{fas} = \frac{5 \times 0.05}{V_{fas}} \qquad [2.1]$$

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:

$$COD = \frac{(A - B) \times M_{fas} \times 8{,}000}{V} \quad [2.2]$$

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), and

8,000: milliequivalent weight of oxygen x 1,000 mL/L.

2.1.2. Total Organic Carbon (TOC)

Organic carbon in liquid samples may include a variety of organic compounds in different oxidation states. Total Organic Carbon (TOC) is a more convenient and direct expression of total organic content than COD, but does not provide the same information. Unlike COD, TOC is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen and hydrogen, and inorganics that can contribute to the oxygen demand measured by COD (APHA-AWWA-WPCF, 1999). To determine the quantity of organically bound carbon, the organic molecules must be broken down and converted to a single carbon molecular form that can be measured quantitatively. The TOC concentration was determined by a Shimadzu analyzer (TOC-5000) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentration. The instrument is connected to an automated sampler (Shimadzu, ASI-5000-S). TC is determined from the amount of CO_2 produced during the combustion of the sample at 680°C, using platinum immobilised over alumina spheres as catalyst. The IC is 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 infrarred analyzer (NDIR) after being cooled and dried. High purity air is used as carrier gas with a

flow of 150 mL/min. 4-points calibration curve in the range of 0-1 g C/L, using potassium phthalate as standard for TC and a mixture of sodium carbonate and bicarbonate (Na_2CO_3/$NaHCO_3$, 3:4 w/w) for IC, is used for the quantification (Figure 2.1).

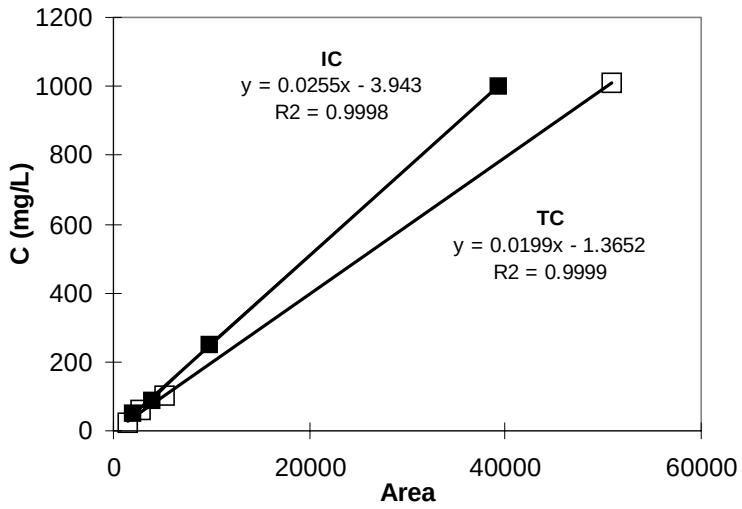


Figure 2.1. Calibration curve

2.1.3. Volatile Fatty Acids (VFA)

Volatile Fatty Acids (VFA), acetic, propionic, i-butiric, n-butiric, i-valeric and n-valeric, are intermediate products of the anaerobic digestion. A VFA accumulation reflects a kinetic disequilibrium between the acids producers and the acids consumers (Switzembaum *et al.*, 1990) and it is an indicator of process destabilization.

VFA are determined by gas chromatography (HP, 5890A) equipped with a Flame Ionization Detector (FID) and an automatic injector (HP, 7673A). The determination is performed in a glass column (3 m long and 2 mm of internal diameter) filled with Chromosorb WAW (mesh 100/120) impregnated with NPGA (25%) and H_3PO_4 (2%). The column, injector and detector temperatures are 105, 260 and 280°C, respectively. Gas N_2, previously saturated with formic acid before entering into the injector, is used as carrier gas with a flow of 24 mL/min. Air and H_2 are used as auxiliary gases with flows of 400 and 30 mL/min,

respectively. VFA, after being separated in the column according to their molecular weights, are burnt in a H_2-air flame and finally measured in the FID at 280°C. The quantification of the sample is made with a 6-8 point calibration curve for each acid in the range of 0-1 g/L, using pivalic acid as internal standard (Figure 2.2).

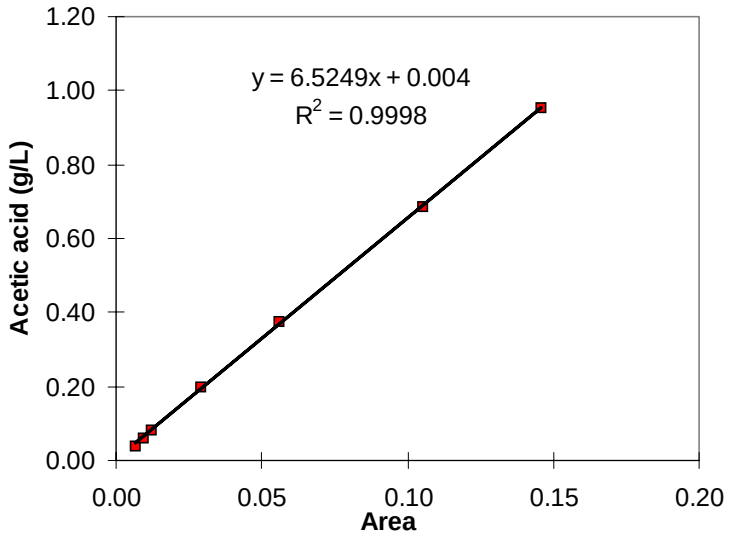


Figure 2.2. Calibration curve for the acetic acid.

2.1.4. Nitrogen

In waters and wastewaters, the forms of nitrogen of greatest interest are, in order of decreasing oxidation state, nitrate, nitrite, ammonia and organic nitrogen. All these forms, as well as nitrogen gas (N_2), are biochemically interconvertible and they are the components of the nitrogen cycle.

Organic nitrogen is defined functionally as the organically bound nitrogen in the trinegative oxidation state, but it does no include all organic nitrogen compounds. Analytically, organic nitrogen and ammonia can be determined together and have been referred to as “Total Kjeldahl Nitrogen” (TKN), a term that reflects the technique used in their determination.

Total inorganic nitrogen (IN) is the sum of the nitrate and nitrite forms.

Total (TN), Inorganic (IN) and Total Kjeldhal Nitrogen (TKN)

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

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

Ammonia nitrogen

Ammonia nitrogen is determined by a colorimetric method (Wheatherburn, 1967). It is based on the reaction of NH_3 with HClO and phenol, forming a strong-blue compound (indophenol) which can be colorimetrically determined using a spectrophotometer (Shimadzu UV-1603, UV-Visible) at 635 nm.

Reagents preparation

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

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

Determination procedure

Place 2.5 mL of sample (diluted if necessary to get a maximum concentration of 1 mg NH_4^+-N/L) and add, 1.0 and 1.5 mL of solution 1 and 2, respectively. After waiting 45 min at room temperature, the concentration of NH_4^+-N is measured in a spectrophotometer at 635 nm. The quantification is done with a 5-7 points calibration curve in the range of 0-1 mg NH_4^+-N/L, using NH_4Cl as standard (Figure 2.3).

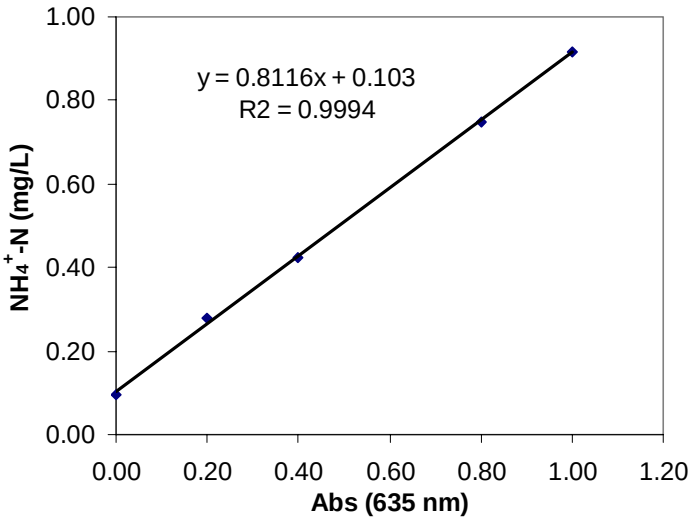


Figure 2.3. Calibration curve

Nitrite

Nitrite concentration in wastewater is determined following the method 4500-NO_2^--B described in *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 1999).

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

Reagents preparation

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

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

Determination procedure

To 5 mL of sample (diluted if necessary to fit the concentration range of the method), 0.1 mL of each solution (sulphanilamide and NED) are added. After waiting 20 min for colour stabilisation, the sample is measured in a spectrophotometer (Shimadzu UV-1603) at 543 nm. The quantification is done with 6-8 points calibration curve in the range of 0-0.30 mg NO_2^--N/L, using $NaNO_2$ as standard (Figure 2.4).

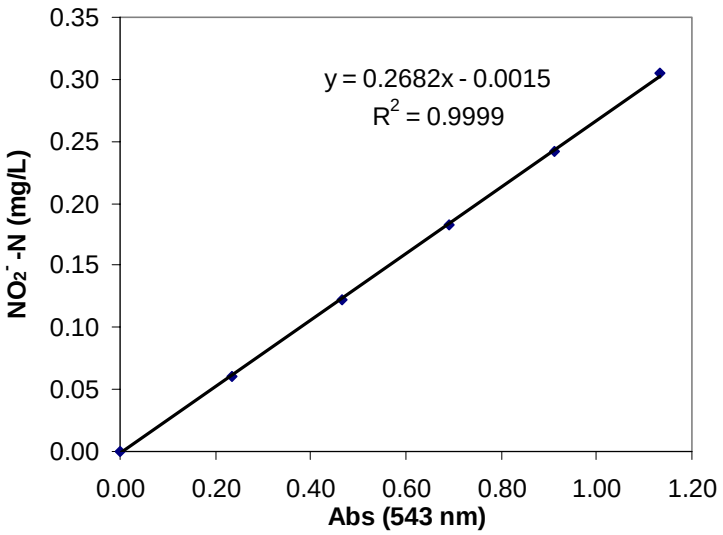


Figure 2.4. Calibration curve

Nitrate

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

Measurement of UV absorption at 220 nm enables rapid determination of NO_3^- ions. Because dissolved organic matter also may absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement at 275 nm is used to correct the NO_3^- value.

Determination procedure

Place 5 mL of sample (diluted if necessary to get a maximum concentration of NO_3^--N of 2.5 mg/L) and add 0.1 mL of HCl 1N. Afterwards, the absorbance at 220 and 275 nm is measured in a spectrophotometer (Shimadzu UV-1603). The absorbance related to nitrate is obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm. The quantification is done with a 6-8 points calibration curve in the range of 0-2.50 mg NO_3^--N/L, using KNO_3 as standard (Figure 2.5).

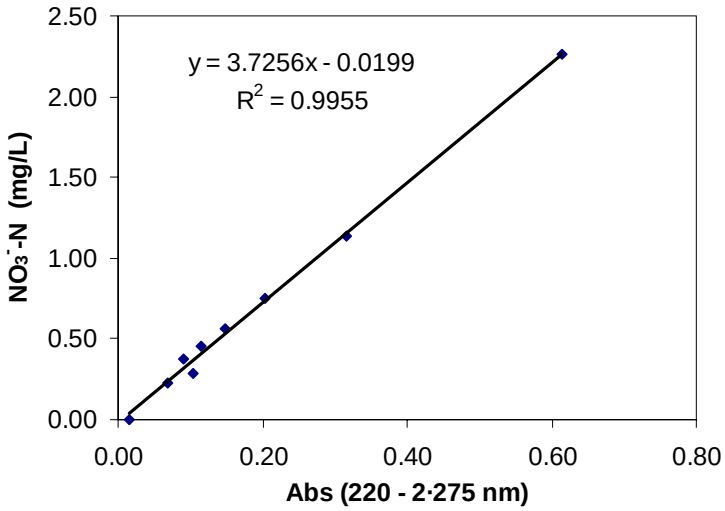


Figure 2.5. Calibration curve

2.1.5. Inorganic anions: NO_2^-, NO_3^-, Cl^-; PO_4^{3-} and SO_4^{2-}

Nitrite (NO_2^-), nitrate (NO_3^-), chloride (Cl^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) are determined simultaneously by Waters Capillary Ion Analyzer (CIA). A solution of sodium cromate (0.005 mol/L) is used as electrolyte (Vilas-Cruz *et al.*, 1994). Plus, an electro-osmotic modifier (50 mL/L) CIA-Pak™ OFM Anion BT

Waters (Ewing *et al*., 1989; Heiger, 1992) is also added. The sample is forced to migrate through a capillary (melting silica covered with poliimida, 60 cm long and 75 μm of internal diameter) kept at 25°C by the application of an electric current. Depending on the ratio charge/mass of the ion, the migrating time is different. A hydrostatic injection (10 cm height for 30 seconds) and an indirect detection (UV, 254 nm, 20 kV, 16-22 μA) are used.

4-6 calibration points for each ion in the range of 3-100 $mg \cdot L^{-1}$ are daily used for the quantification of the samples (Figure 2.6).

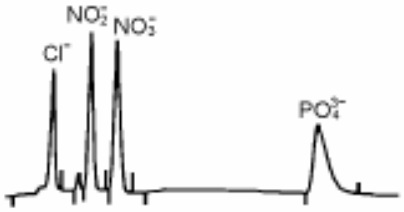


Figure 2.6. Typical electropherogram

2.1.6. Other control parameters

pH

pH is one of the key parameters used in wastewater treatment, since its control is important to maintain the biological activity of the microorganisms involved in the treatment process.

pH measurements were performed with an electrode (Crison Instruments, S.A., 52-03) equipped with an automatic compensatory temperature device (Crison Instruments, S.A., 21-910-01) and connected to a measure instrument (pH/mV). The sensibility of the instrument is ± 1 mV, corresponding to 0.01 pH units. The electrode is calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

Dissolved oxygen (DO)

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

Temperature

A probe (Thermometer checktemp1, model Celsius HI98509 HANNA instruments) was used to determine the temperature.

2.2. Solid phase

2.2.1. Total and Suspended Solids

Solids present in water can be organic or inorganic. Total Solids (TS), Total Suspended Solids (TSS), Volatile Solids (VS) and Volatile Suspended Solids (VSS) are determined following the methods 2540B, 2540D and 2540E, respectively, described in *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 1999).

Determination procedure

TS 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) dish 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 TSS, a selected (in order to yield a residue between 2.5 and 200 mg) well-mixed sample volume is filtered through a weighed glass-fiber 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 (VS or VSS), the residue from method 2540B and 2540D is burnt to constant weight at 550ºC during half an hour. The weight lost during ignition corresponds to the volatile solids. Since only a small amount of inorganic salts are decomposed and volatilised at that temperature.

This determination is useful in the control of wastewater treatment plan 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.

2.3. Biomass characterisation

2.3.1. Granules density

The biomass density (as mass of granules per volume of granules) was determined with dextran blue using the method described by Beun (Beun *et al.*, 2002**).** A known amount of sample is taken from the reactor. The mixture is mixed and the granules are allowed to settle. A known amount of the liquid above the settle granules is removed and a sample is taken from it. A known volume of a dextran blue solution (1 g/L) is added to a representative sample (and known amount) of granular sludge, in a volume ratio of about 1:1. The mixture is gently mixed, and subsequently the granules are allowed to settle. A known amount of the liquid above the settled granules is removed and a sample is taken from it (fraction 1). This fraction and the original dextran blue solution are analyzed by a spectrophotometer at 620 nm. Subsequently the volume occupied by the biomass in the reactor sample is calculated, since dextran blue only binds to the water and not to the biomass. Measuring also the dry weight of the reactor sample (using the previously described methodology) allows calculation of the density of the granules as g biomass per L of granules.

The density is calculated with the following equation:

$$\text{Density} = \frac{Vinitial \times \text{VSS}}{\text{Vbiomass}} \qquad [2.3]$$

where:

[VSS]: Volatile Suspended Solids (g/L),

Vinitial = P_2 - P_1

Vbiomass = P_4-P_1-V_L

P_1: test tube weight

P_2: weight of the test tube with sample

P_3: weight of the test tube with sample after liquid above is removed

P_4: weight of the test tube after add dextran blue

V_L is calculated with the following equation:

$$VL = \frac{Ainitial \times Vdextran}{Afinal} \quad [2.4]$$

Where:

Ainitial: Absorbance of the dextran blue solution (1 g/L)

Afinal: Absorbance of the sample

Vdextran: is calculated as the difference between P_4 and P_3

2.3.2. Average diameter of the granules

Changes in morphology of the granules were followed by image analysis (IA) (Jeison and Chamy, 1998; Tijhuis *et al*., 1994).

Images of the granular sludge were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (SMZ-2T, Nikon and Stemi 2000-C, Zeiss, respectively).

For digital image analysis the programme Image ProPlus was used. Specifically the programme served to calculate the mean feret diameter of the granules. It has to be noted that in cases where not enough granules had been caught in the analysed pictures representatively might be critical.

The feret diameter is calculated as an average value from the shortest and the longest measured segment in the granule. (Figure 2.7)

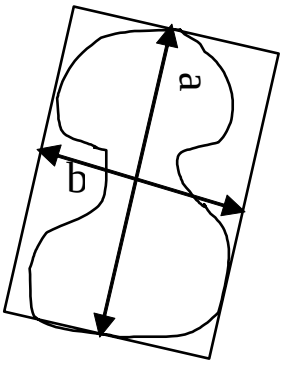


Figure2.7. Shortest and longest segments in a granule.

2.3.3. Electron Microscopy and Micro-analysis

Morphological studies of the biomass were performed with a scan electron microscope (Digital SEM Leica 440 at 20 kV) controlled with a computer system and with a magnification capacity ranging from 15 to 290000 folds. The sludge sample was washed three times for 10 minutes with phosphate buffer 0.05 N at pH 7.4 and subsequently fixed with a solution of glutaraldehyde 3% in phosphate buffer over night. After fixation the sample was dehydrated using ethanol solutions with increasing ethanol concentrations (30, 50, 70 and 100 %). Later the sample was shaded with gold and observed under the scan electron microscope. To investigate the elemental composition of the granules micro-analysis was carried out. The instrument used was the SEM LEO-435VP with system of micro-analysis (EDX) at varying voltages 5 kV, 20 kV and 30 kV.

2.3.4. Sludge Volumetric Index

The Sludge Volumetric Index (SVI) was determined according to the procedure specified in the Standard Methods for the Treatment of Water and Wastewater (APHA, 1999). The VSI is the volume in millilitres occupied by 1 g of a suspension after 30 min settling. SVI typically is used to monitor settling characteristics of activated sludge and other biological suspensions. Although SVI is not supported theoretically, experience has shown it to be useful in routine process control.

For the determination of VSI was required determined the suspended solids concentration of a well-mixed sample of the suspension. Then, it was determined the 30 min settled sludge volume and finally the SVI according to the equation 2.5.

$$SVI = \frac{Settled\ sludge\ volume(mL/L) \times 1000}{Suspended\ solids(mg/L)} \qquad [2.5]$$

Besides, at the "*1st IWA-Workshop Aerobic Granular Sludge*" (Munich, 2004) where aerobic granules were defined (de Kreuk *et al*., 2005) was also defined another parameter SVI_{10}.

This means that SVI_{10} (SVI after 10 minutes of settling) in combination with SVI_{30} should be used for characterizing the settleability of granular activated sludge as was suggested by Schwarzenbeck *et al.* (2004). Both values should be reported in papers written about aerobic granules, since the difference between the SVI_{10} and SVI_{30} value gives an excellent indication about the granule formation. Sieving is considered a proper method to harvest granules from activated sludge tanks or from aerobic granule reactors.

2.3.5. Zone Settling Velocity

The Zone Settling Velocity (ZSV) was determined according to the procedure specified in the Standard Methods for the Treatment of Water and Wastewater (APHA, 1999). At high concentrations of suspended solids, suspensions settle in the zone-settling regime. This type of settling takes place under quiescent conditions and is characterized by a distinct interface between the supernatant liquor and the sludge zone. The height of this distinct sludge interface is measured with time.

Determination procedure

Record height of solids-liquid interface at intervals of about 1 min. Collect data for sufficient time to assure that suspension is exhibiting a constant zone-settling velocity and that any initial reflocculation period, characterized by an accelerating interfacial settling velocity, has been passed.

Zone settling rate is a function of suspended solids concentration and suspension height. Plot interface height in centimetres versus time in minutes (Figure 2.8). Draw straight line through data points, ignoring initial shoulder or reflocculation period and compression shoulder. Calculate interfacial settling rate as slope of line in centimetres per minute.

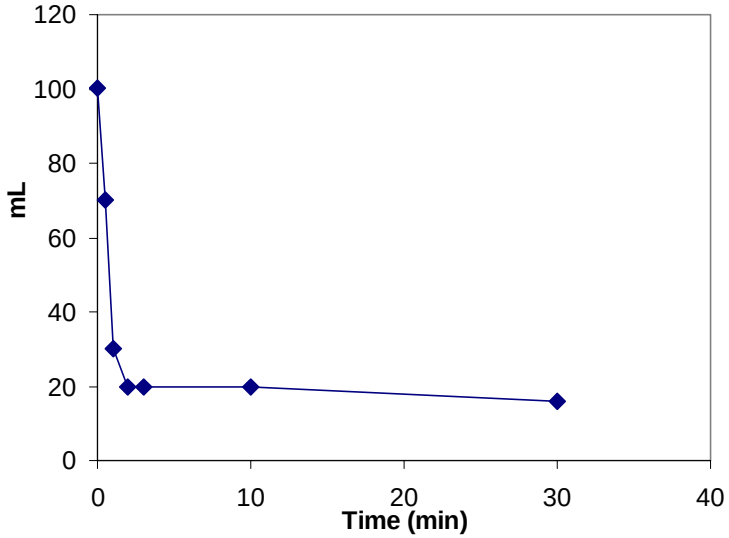


Figure 2.8. Millilitres settled versus time

2.4. Microbiological determinations

2.4.1. Faecal coliforms and *Escherichia Coli*

Faecal coliforms and *Escherichia Coli* were measured with a membrane filtration technique using chromogenic *E.Coli*/coliform (C-EC Agar) media. The obtained results were expressed as Coliform Forming Units (CFU) in 100 mL/sample (Isra-Cnr, 1994) (Figure 2.9 and 2.10). *E. Coli* was enumerated from faecal coliforms by using the Wood Lamp.

Determination procedure

1. Growth media preparation

The composition of the chromogenic *E.Coli*/coliform (C-EC) growth media (Biolife), is detailed in Table 2.1. The media was prepared fresh and heated to the boiling point and then sterilized in the autoclave, at a temperature of 120 ºC and at pressure (1 atm), during 15 minutes. A volume of 14 mL of liquid media obtained after sterilization was placed in each sterile Petri plate (9 cm of diameter and 1.5 cm of height), which were stored in the fridge at 4 ºC after the media solidified.

Tabla 2.1. Media composition C-EC Agar (Isra-Cnr, 1994).

Compounds	Values (g/L)
Tryptose	10
Peptocomplex	5
Yeast extract	3
Sodium chloride	5
PTG	0.10
X-GAL	0.08
MUG	0.05
Agar Bios 1L	1.3

2. Filtration and incubation

For the bacteria determination, the samples were filtered in triplicate using membranes (of cellulose nitrate) of 47 mm of diameter with a pore size of 0.45 μm (Sartorius) (Figure 2.9),

An amount of 100 mL of sample was filtered by means of a vacuum system using sterile material and a steel support, which was also sterilized. After the filtration, the filter was placed on the Petri plates containing the media. The filter was incubated in a heater for 24 h at 44 ºC.

3. Enumeration of the colonies

During the permanence time in the heater, with the ideal media and temperature, each single bacteria present in the sample multiplies forming a colony, which results visible. The number of colonies formed on the plate is counted. For the determination of faecal coliform, all blue coulored colonies were counted.

The faecal coliforms were directly counted (blue colonies); however, *Escherichia Coli* bacteria distinguished from faecal coliforms using a fluorescence lamp (wood lamp). All the results were expressed as Coliform Forming Units (CFU) per 100 millilitres of sample (Figure 2.10).

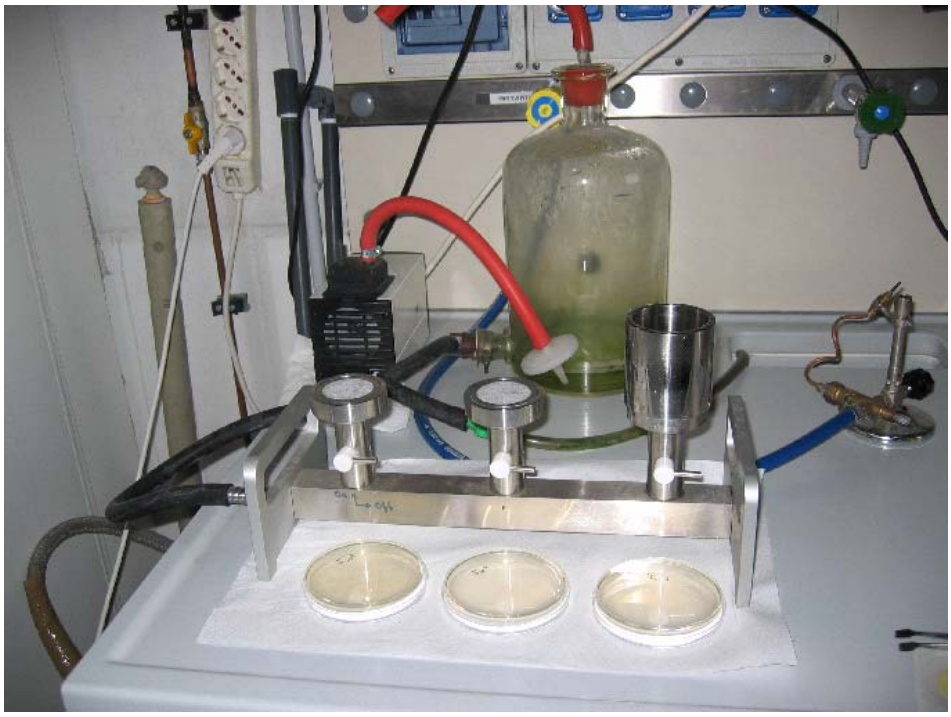


Figure 2.9. Membrane filtration device.

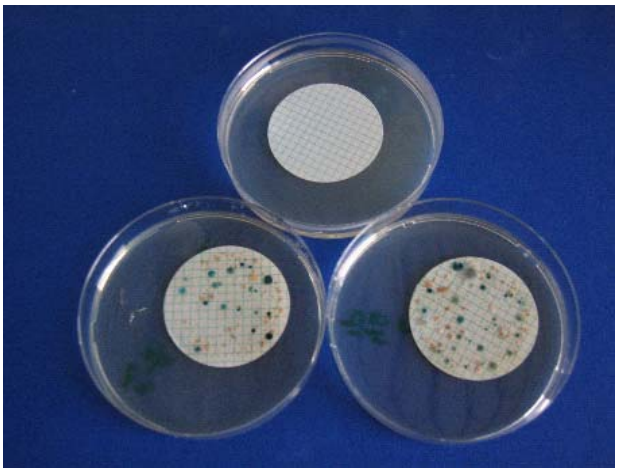


Figure 2.10. Enumeration of the colonies.

2.4.2. Identification of bacteria populations by Fluorescent in Situ Hybridisation

The abundance of the different populations of microorganisms present in the sludge samples of the reactors was researched by Fluorescent In Situ Hybridization (FISH). Figure 2.11 shows the phylogenetic tree reflecting the relationships between different bacteria: ammonia-oxidizing (AOB) and nitrite-oxidizing bacteria (NOB). The phylogenetic tree for *Plantomycetales* and other reference organisms is shown in Figure 2.12.

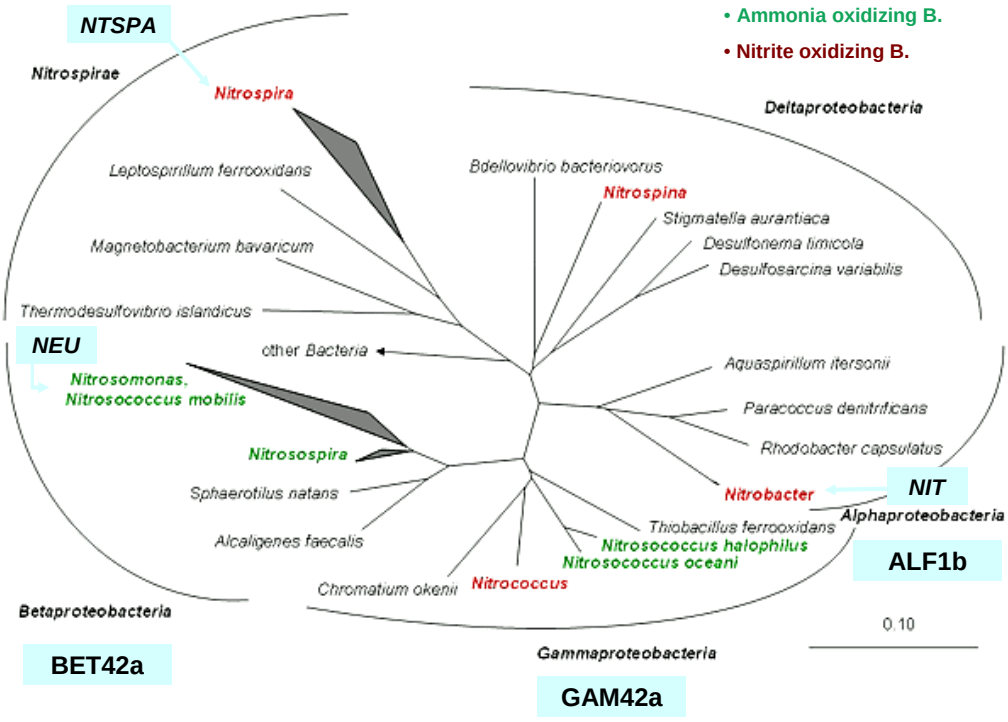


Figure 2.11. 16S rRNA-based tree showing the phylogenetic affiliation of AOB (green) and NOB (red). The scale bar indicates 0.1 estimated change per nucleotide.

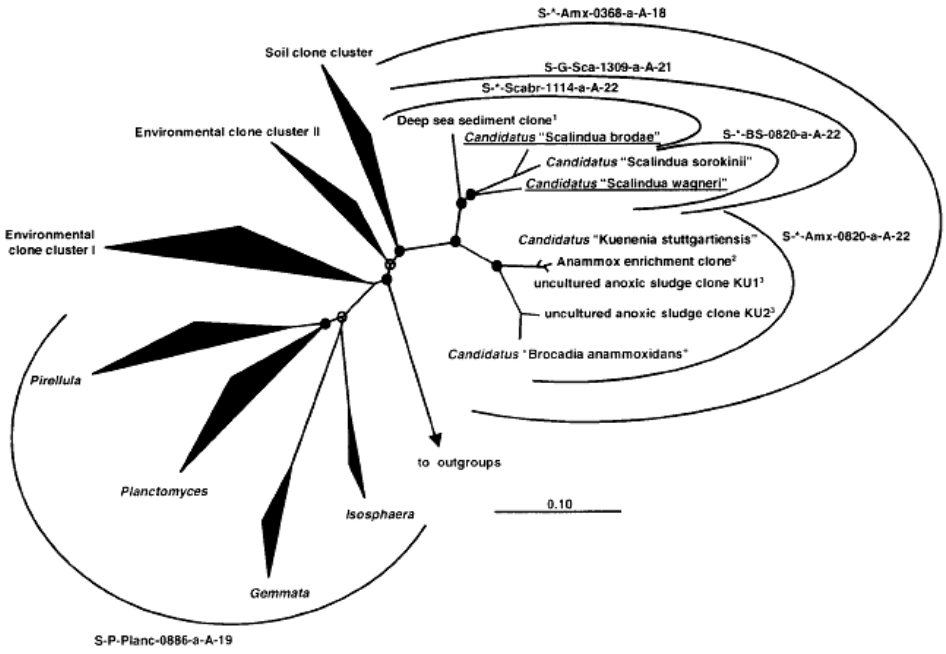


Figure 2.12. 16S rRNA-based tree showing the phylogenetic affiliation of *Plantomycetales*. The scale bar indicates 0.1 estimated change per nucleotide.

In this technique specific regions in 23S or 16S rRNA are targetted with fluorescently labelled probes. If the corresponding domain, phylum, genus or species is present, the probe hybridises to the target sequence and can later be detected microscopically. According to Amann (1995) a typical FISH protocol includes four steps (Figure 2.13): the fixation and permeabilisation of the sample; hybridisation of the target sequence to the probe; washing steps to remove unbound probe; and the detection of labelled cells by microscopy or flow cytometry. The method followed in this study is detailed in Amann (Amann *et al.*, 1990).

During hybridization the cells are exposed to elevated temperatures, detergents and osmotic gradients. Thus fixation of the cells is essential in order to maintain the morphological integrity of the cells. Fixation of cells with glutaraldehide results in considerable autofluorescence of the specimen.

Autofluorescence is minimized by fixation in freshly prepared (not older than 24 h) 4% paraformaldehyde solution in PBS.

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

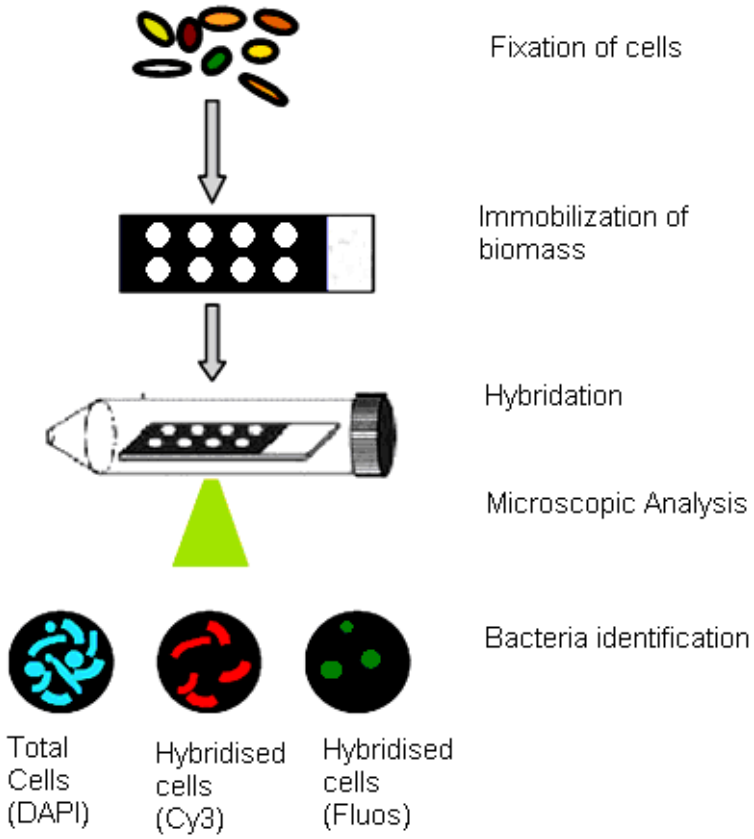


Figure 2.13. Typical FISH protocol

The dyes used to detect the hybridised rRNA were fluorescein (λexc. = 495nm, λem. = 519nm) and carbocyanine (λexc. = 558nm, λem. = 670nm). To

visualise all cells in a sample the stain DAPI was used (λexc. = 359nm, λem. = 461nm). Its application can provide insight into the existence of archaeo-bacteria and eukaryotes, like e.g. protozoa.

For analysis of the slides an epifluorescence microscope (Axioskop 2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics) was used. The probes applied in this study are listed in Table 2.2.
The three probes for the domain of eubacteria (EUB338, EUB338II and EUB338III) 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.

Probe PLA46 was used in combination with Amx820 to eventually provide evidence of the existence of Anammox bacteria (or other planctomycetales) other than those covered by Amx 820.

In some cases probe Nso 1225 was combined with NEU 653. Besides NEU653 and Ntspa712 were used simultaneously for the characterisation of the structure of the nitrifying population.

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 irrepresentative sampling as well. Still it was tried to keep this error small. It has to be noted that when the term Anammox organisms, ammoniumoxidisers or nitrite-oxidisers is used, it refers to those staining with the corresponding probes, i.e. Amx 820, NEU 653 and Ntspa 712, respectively. The denomination EUB refers to all three EUB-probes.

Probe-name	Target site 16S	Probe sequence (5'→3')	% Fornamide in situ	Target organisms	Ref.
EUB338	338-355	GCT GCC TCC CGT AGG AGT	35	Bacteria domain	Amann *et al*., 1990
EUB 338-II		GCA GCC ACC CGT AGG TGT	60	Bacterial lineages not covered by probe EUB338	*Daims et al., 1999*
EUB 338-III		GCT GCC ACC CGT AGG TGT	60	Bacterial lineages not covered by probe EUB338 and EUB338II	*Daims et al., 1999*
PLA46	46-63	GAC TTG CAT GCC TAA TCC	20	Planctomycetes	Neef *et al*., 1998
NEU653 (& competitor)	653-670	CCC CTC TGC TGC ACT CTA TTC CAT CCC CCT CTG CCG	40	Most halophilic and halotolerant Nitrosomonas spp.	Wagner *et al*., 1995
Nso1225	1225-1244	CGC CAT TGT ATT ACG TGT GA	35	Ammonio-oxidizing-β- *Proteobacteria*	Mobarry *et al*., 1996
Nsv443	444-462	CCG TGA CCG TTT CGT TCC G	40	*Nitrosolobus multiformis*, *Nitrosospira briensis*, and *Nitrosovibrio tenuis*.	Mobarry *et al*., 1996
NIT1035 (& competitor)		CCT GTG CTC CAT GCT CCG CCT GTG CTC CAG GCT CCG	40	*Nitrobacter* spp.	Wagner *et al*. ,1996
Ntspa712 (& competitor)	712-732	CGC CTT CGC CAC CGG CCT TCC CGC CTT CGC CAC CGG GTT CC	35	Most members of phylum *Nitrospira*	Daims *et al*., 2001
AMX820	820-841	AAA ACC CCT CTA CTT AGT GCC C	35	*Candidatus* "Brocardia anammoxidans"	Schmid, 2001
Alf1b	19-35	CGT TCG YTC TGA GCC AG	20	Alphaproteobacteria, some Deltaproteobacteria, Spirochaetes	Manz *et al*., 1992
Gam42a	1027-1043	GCC TTC CCA CAT CGT TT	35	Gammaproteobacteria	*Manz et al., 1992*
NSm156	156-174	TAT TAG CAC ATC TTT CGA T	5	Nitrosomonas spp., Nitrosococcus mobilis	*Mobarry et al.,1996*
Bet 42a	1027-1043	GCC TTC CCA CTT CGT TT	35	Betaproteobacteria	Manz *et al*., 1992

Table 2.2. Probes used for fluorescent in situ hybridisation and the formamide concentration used during hybridisation

2.5. References

Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flowcytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, **56**, 1919–1925.

Amann R.I. (1995). In situ identification of micro-organisms by whole-cell hybridization with RNA-targeted nucleic acid probles, p.1-15. *In A.D.L.: Akkerman, J.D., van Elsas and F.J. de Brujin (ed), molecular microbial ecology manual. Kluwer Academic Publisher*, Dordrecht, The Netherlands.

APHA-AWWA-WPCF. (1999). Standard Methods for the examination of water and wastewater. 20th Edition. Clesceri, L.S., Greenberg, A.E. & Eaton, A.D. (eds.).

Beun J.J., van Loosdrecht M.C.M. and Heijnen J.J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research*, **36**, 702-712.

Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, **22,** 434-444.

Daims H., Nielsen J. L., Nielsen P. H., Schleifer K. H. and Wagner M. (2001). In Situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*, **67**, 5273-5284.

de Kreuk M.K., McSwain B.S., Bathe S., Tay S.T.L., Schwarzenbeck and Wilderer P.A. (2005). Discussion outcomes. Ede. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing*. Munich, (165-169).

Ewing, A.G., Wallengford, R.A. and Oleferowicz, T.M. (1989). Capillary electrophoresis. *Analytical Chemistry*, **61** (4), 292A-303A.

Heiger, D.N. (1992). High performance capillary electrophoresis: an introduction. Hewlett Packard GmbH, Waldbronn, Germany.

Isra-Cnr (1994). “Metodi analitici per le Acque”.

Jeison D. and Chamy R. (1998). Novel technique for measuring the size distribution of granules from anaerobic reactors for wastewater treatment. *Biotechnology Techniques*, **12** (9), 659-662.

Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Systematic and Applied Microbiology*, **15**, 593 - 600.

Mobarry, B., M. Wagner, V. Urbain, B. Rittmann, and D. Stahl. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology*, **62**, 2156–2162.

Neef A., Amann R., Schlesner H. and Schleifer K.-H. (1998). Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology*, **144**, 3257-3266.

Schmid M., Schmitz-Esser S., Jetten M. and Wagner M. (2001). 16S-23rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental Microbiology*, **3** (7), 450-459.

Soto, M., Veiga, M.C., Méndez, R. and Lema, J.M. (1989). Semi-micro COD determination method for high salinity wastewater. *Environmental and Technology Letters*, **10** (5), 541-548.

Switzembaum, M.S., Giraldo-Gómez, E. and Hickey, R.F. (1990). Monitoring of the anaerobic methane fermentation process. *Enzyme and Microbial Technoogy*, **12** (10), 722-730.

Schwarzenbeck N., Erley R. and Wilderer P.A. (2004). Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter. *Water Science and Technology*, **49** (11-12), 41-46.

Tijhuis L., van Benthum WAJ, van Loosdrecht MCM and Heijnen J.J. (1994). Solids retention time in spherical biofilms in a biofilm airlift suspension reactor. *Biotechnology Bioengineering*, **44**, 867-879.

Vilas-Cruz, M., Gómez, J., Méndez, R. and Lema, J.M. (1994). Determinación simultánea de NO_2^- y NO_3^- en aguas residuales por electroforesis capilar. *Proc.*

of the III International Symposium of Analytical Methodology for the Environment. Vol. **II**, 1-50. Barcelona, 23-24 March.

Wagner M., Rath G., Koops H.P., Flood J. and Amann R. (1996). In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*, **34**, 237-244

Wheatherburn M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry***, 28**, 971-974.

Chapter 3

Aerobic granulation with industrial wastewater in sequencing batch reactors[1]

Summary

Granular sludge formation was promoted in two laboratory scale sequencing batch reactors (SBR), R1 and R2 fed with industrial wastewater produced in a laboratory for analysis of dairy products. Both reactors were operated under similar conditions during most of the experimental period. However, an anoxic phase between 10 and 30 min was included at the beginning of every cycle of operation of R1, but not in R2. Organic and nitrogen loading rates (OLR and NLR) applied to both systems were high, up to 7 g COD/(L·d) and 0.7 g N/(L·d). Nitrogen removal efficiency was 70% in both units even considering that R2 was operated always under aerobic conditions. Granules with similar morphology were developed in both systems. Granular size distribution was comprehended between 0.25 and 4.0 mm for both systems.

The presence of TSS in the effluent of the SBRs was strongly affected by either the length of the withdrawal period or by the applied particulated COD to biomass ratio (CODp/VSS) to the systems. The lower concentrations of TSS in the effluent were attained when the systems operated with an CODp/VSS ratio lower than 0.12 g COD/gVSS. There was a strong reduction of the average TSS content in the effluent from 450 to 200 and 150 mg TSS/L when the length of the withdrawal period was diminished sequentially from 3 to 1 and 0.5 min, respectively. This was caused by a more intensive washout of small suspended biomass aggregates that took place when the length of this period was shortened.

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3.1. Introduction

The performance of the aerobic activated sludge process depends on the settling characteristics of the sludge generated in the reactor. The flocs in the sludge should be easily separated from the treated wastewater in the settler and recirculated to the reactor. However, in some cases flocs with poor settling properties were developed in the system, which might result in partial sludge washout and a decrease of the quality of the effluent. Most of the times this washout is caused by an excessive growth of filamentous microorganisms (Wanner *et al*., 1998) or development of flocs with a fluffy structure, which have a negative influence on the settling properties of the sludge. The control of the causes responsible of the production of sludge with poor settling properties is sometimes difficult. In some cases a change in either the reactor configuration or the operating strategy has influenced the characteristics of the developed sludge in these units. However, for some wastewater the formation of flocs with good settling properties is generally difficult (Blackall *et al*., 1991; Pujol *et al*., 1991; Ng *et al*., 2005). For these reasons, the research on the production of granular biomass as an alternative to promote better biomass retention becomes interesting. Granules have good settling properties, due to the achievement of biomass aggregates with a high biomass density. Besides, the good settling characteristics of the granular sludge improves the separation of biomass from the treated wastewater, which is reflected either in a lower area requirement for settling in continuous units or allowing longer time for biological purification in discontinuous units as SBRs that used granular biomass.

Granular sludge can be generated in upflow anaerobic sludge blanket (UASB) reactors (Lettinga *et al*., 1993). It is generally thought that the upflow velocity in an UASB creates a selective pressure to which the organisms have two responses: to be washed out or to stick together and form easily settleable granules (Liu *et al*., 2003). The formation of granular sludge was also obtained in aerobic sequencing batch reactors (SBR) fed with synthetic or industrial wastewater (Tay *et al.*, 2002a; Etterer and Widerer, 2001; Liu and Liu, 2006; Hailei *et al*., 2006). In SBRs, the wastewater is treated in successive cycles of a few hours. At the end of every cycle, settling of the biomass takes place before

the effluent is withdrawn, to keep the biomass in the reactor. There is evidence that the basis of granulation is the continuous selection of sludge particles that occurs inside the reactors. The part of the biomass, which does not settle fast enough, will be washed out with the effluent (Beun *et al.*, 1999). Thus, the selection of the granules from a biomass mixture in an SBR can be easily performed using the difference in settling velocity between the granules (fast settling biomass) and the flocs (slow settling biomass). Because the settling velocity is an important selection criteria, utilisation of either relatively large column height diameter (H/D) ratio or short settling and water withdrawal periods is advantageous. Additionally, the formation of granules is favoured when time of feeding period is reduced to some minutes per cycle (Beun *et al.*, 1999). They have also pointed out that the promotion of slow growing biomass able to store compounds increases the density of the formed granules. On the other hand, high hydrodynamic shear forces seem to stimulate the production of cellular polysaccharides, as it has also been observed in the case of biofilms. It is therefore reasonable to conclude that the formation and stability of aerobic granules are dependent on the hydrodynamic shear force-associated cellular polysaccharides production (Tay *et al.*, 2001a; Tay *et al.*, 2001b; Wang *et al.*, 2006). As compared with conventional activated sludge flocs, heterotrophic granular sludge has regular, dense and strong microbial structures, good settling properties, high biomass retention, and the ability to withstand shock loads (Tay *et al.*, 2002b).

Granulation could be a solution for operating some SBRs in which flocculent sludge with poor settling properties is developed. During a previous research, it was found that the settling properties of the sludge generated in an industrial scale SBR, with high COD and nitrogen efficiencies, were poor as the solids volume index (SVI) was never lower than 100 mL/gSST (Garrido *et al.*, 2001) and the zone settling velocity (ZSV) was around 0.3 m/h.

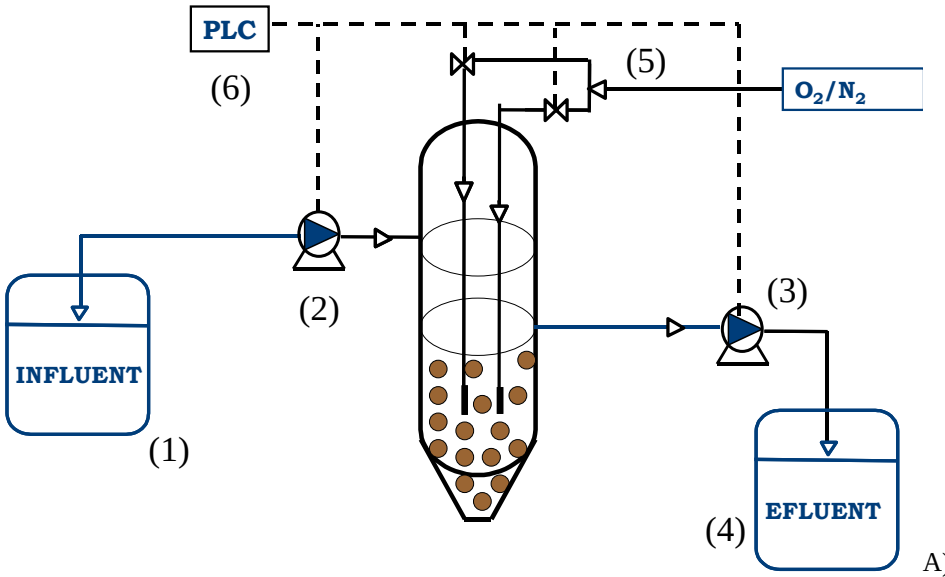
3.2. Objectives

In order to study the feasibility of obtaining granules in the industrial scale SBR, two laboratory scale SBRs were seeded with the sludge collected from the industrial scale SBR, fed with the same influent used in this unit and operated with several conditions to promote granules formation. An additional objective was to study the nitrogen removal in these granular systems and to investigate the causes that could influence the suspended solids content in the effluent. The formation of granules under different conditions was studied and diameter, density and final settling velocity were examined.

3.3. Materials and methods

3.3.1. Experimental set-up

Two sequencing batch reactors (SBR) with a total volume of 2.5 L and a working volume of 1.5 L were used. Dimensions of the units were: height of 465 mm and inner diameter of 85 mm. The height to the diameter ratio (H/D) being 5.5. The maximum level of the liquid was 264 mm, and the minimum level of 132 mm after effluent withdrawal. Oxygen was supplied to both reactors by using spargers to promote the formation of small air bubbles. In case of R1 air was replaced temporarily by nitrogen gas during an anoxic period comprehended between 10 and 30 min. The flow of nitrogen and air was controlled by means of two electrovalves. A set of two peristaltic pumps was used to feed and to discharge the effluent, respectively, in both reactors. The influent was introduced in both systems through ports located at the top of the reactors. The effluent was discharged through the sampling port placed at middle height of the column reactor. A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations over the pumps and valves, and thus the length of every operational period in the SBRs (Figure 3.1). The reactor was operated at room temperature (15-20 ºC) and at oxygen concentration between 0 (anoxic period) and 4-8 mg O_2/L (oxic period) and without pH control, which varied between 7.4 and 8.5.



A)

B)

Figure 3.1. A)Experimental set up: (1) Feeding tank; (2) Feeding pump; (3) Effluent pump; (4) Effluent tank (5); Air and N_2 valves; (6) PLC. B) Picture of the reactor.

3.3.2. Inocula

Both reactors were inoculated with sludge collected from the industrial SBR. This sludge was the typical flocculent activated sludge with a fluffy, irregular and loose morphology and relative abundance of filamentous microorganisms. Settling properties of this sludge were: SVI of 200 mL/g VSS, and ZSV of 0.3 m/h.

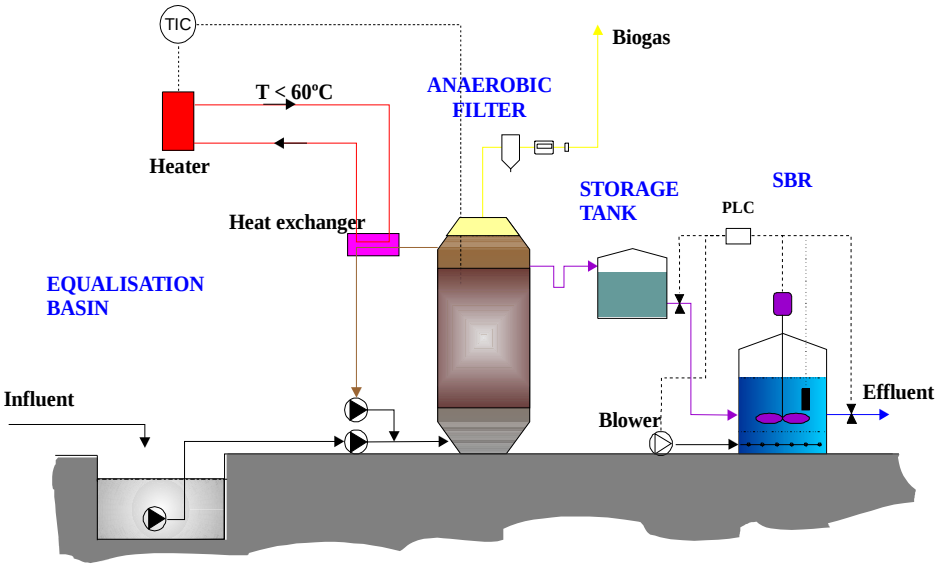
3.3.3. Feeding media

Two different feedings were used during the studies with both SBRs: a synthetic wastewater and an industrial wastewater from a laboratory for analysis of dairy products located near A Coruña, Spain (Table 3.1). The synthetic wastewater contained soluble COD as the sole organic matter fraction and the industrial wastewater contained both soluble and particulated COD. The composition of the synthetic wastewater (SW) fed to reactor R1 was according to Beun *et al*. (1999) and the trace solution to Smolders *et al*. (1995). The industrial wastewater was the same as that used to feed the industrial scale SBR of 28 m^3 and was previously treated in an anaerobic filter in order to reduce the organic matter fraction (Figure 3.2). Additional information about the generation of the industrial wastewater and of the treatment plant can be found elsewhere (Garrido *et al*. 2001; Omil *et al*. 2003; Arrojo *et al*., 2003; Fernández *et al*., 2004).

Table 3.1. Composition of wastewater of the laboratory for analysis of dairy products (IW) and the synthetic wastewater (SW) used to feed the SBRs.

Parameters	**Concentration (mg/L) IW**	**Concentration (mg/L) SW**
CODt	500-3000	500
CODs	300-1500	500
Total nitrogen	50-200	25
TSS	200-1200	0
VSS	100-1000	0
VFA	100-500	632[1]
Phosphate	20-60	23

([1] acetate concentration in the SW)



A)

B)

Figure 3.2. Schematic layout of the plant (A) and picture of the industrial wastewater plant (B).

3.3.4. Strategy of operation

The operation of R1 and R2, summarized in Table 3.2, did not commence simultaneously, thus day 1 corresponds in this study to the day when R1 was fed for the first time with the synthetic wastewater for 27 days. On day 27 the synthetic medium was replaced stepwise with a fraction of the industrial wastewater, maintaining the soluble COD concentration nearly constant. After day 48, it was fed exclusively with the industrial wastewater. Operation of R2 started 50 days later and was directly fed with the industrial wastewater. The strategy of operation was similar in both systems from day 50 on.

Table 3.2. Main operational stages of the two SBRs which were performed during the study. SW: Synthetic Wastewater; IW: Industrial Wastewater. [1] Volume of SW and IW which were fed to R1.

Day	R1	R2
1	Start up SW	--------
27	0.75 : 0.25 [1]	--------
34	0.50: 0.50 [1]	--------
41	0.25: 0.75 [1]	--------
48	IW	--------
50	--------	Start up IW
83	Anoxic period = 10 min	--------
133	Anoxic period = 30 min	--------
188	Increase OLR	
	Withdrawal time =1 min	
	Withdrawal time =1/2 min	
	Constant OLR	
	Withdrawal time =1 min	
	Withdrawal time =3 min	
	SW	

Table 3.2 shows some of the main operational stages and changes in the strategy of operation. Both systems were inoculated with sludge collected from the industrial SBR and were operated in cycles of 3 hours with an exchange

volume of 50%. Every cycle comprehended a feeding period of 3 min, a reaction period under anoxic and aerobic conditions of 171 min, a settling period of 1 min, an effluent withdrawal period comprehended between 0.5 and 3 min and an idle period between 2 and 4.5 min (Figure 3.3A). On day 83 an anoxic period of 10 minutes was included in the cycle of R1 and from day 133 on, this increased to 30 minutes until the end of operation of the reactor (day 320) (Figure 3.3B).

Especial attention was paid to two experiments during this research: the study of the influence of withdrawal time and of the influence of TSS in the influent over the efficiency of the reactor. The influence of withdrawal period was studied by modifying its length from 3 min to 1 min and to 1/2 min in two kind of experiments: the first using different OLRs (till day 254); and the second maintaining the OLR constant (from day 254 on). During these experiments the sum of time of the consecutive withdrawal and idle periods was fixed at 5 min.

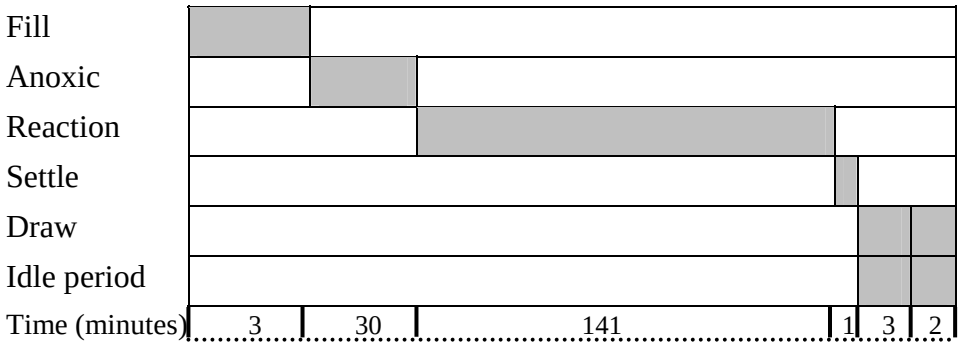
Additionally, in order to study the influence of TSS on the achieved conversions, from day 296 till the end of the experiments (day 320) the units were fed with a synthetic wastewater, which contained soluble COD as the only fraction of organic matter. Data attained in this time interval were compared with those achieved using industrial wastewater.

3.3.5. Analytical methods

The pH, nitrate, ammonia, volatile suspended solids (VSS), total suspended solids (TSS), zone settling velocity (ZSV) and sludge volumentric index (SVI) were determined accordingly to Standard Methods (APHA, 1999) as described in Chapter 2. Concentrations of Chemical Oxygen Demand (COD) were determined by a modified method from the Standard Methods (Soto *et al.*, 1989). Total COD (CODt) was measured directly in the sample and the soluble COD (CODs) from the filtered sample through 0.45 μm pore size filters, particulate COD (CODp) being calculated as the difference between CODt and CODs. The morphology of the granules was measured regularly by using an Image Analysis procedure proposed by Jeison and Chamy counting a sample of more than 200 granules (Jeison and Chamy, 1998). Biomass density, in terms of g VSS per litre of granules, was determined with dextran blue, which is not absorbed by the biomass (Jiménez *et al.*, 1988) and following the methodology proposed by Beun (Beun *et*

al., 1999) according to section 2.3 of Chapter 2. The presence of nitrifying bacteria in granules was followed by Fluorescence *in situ* hybridization (FISH) (Section 2.4 of Chapter2). This analysis was performed with a set of fluorescent labelled 16S rRNA-targeted DNA probes according to the procedure described by Amann (1995). Probes used for fluorescence in situ hybridisation and the formamide concentration used during hybridisation were EUB338 (probe sequence GCT GCC TCC CGT AGG AGT; formamide 35%) and NEU653 (Ammonium Oxidizing Bacteria, AOB) (probe sequence CCC CTC TGC TGC ACT CTA TTC CAT CCC CCT CTG CCG; formamide 40%).

A)



B)

Figure 3.3. Cycle distribution for the SBRs operation. A) R2 and B) R1.

3.4. Results and discussion

In the next section the main results obtained from the operation of both reactors, R1 and R2 are shown in terms of the physical characteristics of the granules, nitrogen and carbon compounds removal and concentration of suspended solids in the effluent.

3.4.1. Biomass granulation process

The sludge used in both systems as inoculum was the typical flocculent activated sludge with a fluffy, irregular and loose morphology and relative abundance of filamentous microorganisms (Figure 3.4A; 3.5A). Settling properties of this sludge were: SVI of 200 mL/g VSS, and ZSV of 0.3 m/h. During the first seven experimental days an almost complete washout of the suspended biomass in both systems was observed. This was a result of the operation strategy of the systems, in which a very short settling and a fast effluent withdrawal period were applied to both reactors. Thus, either flocs or aggregates of biomass with settling velocity slower than 9 m/h were removed from the system as a result of mentioned conditions. Three weeks after the start up of the reactors the formation of small aggregates with an average diameter of 1.05 mm was observed in both systems. Suspended flocs gradually disappeared from the reactor and settling properties of the obtained aggregates were very good, SVI 60 mL/g VSS and ZSV of 20 m/h. Microscopic examination of the sludge showed that the morphology of the granular biomass was completely different from the flocculent sludge that was used as inoculum. The shape of the granules was round with a cauliflower like aspect and very clear outline (Figure 3.4; 3.5)**.**

The granular size distribution along the operational time was monitored and increased gradually with time of operation (Figure 3.6). These results indicated that the formation of aerobic granules was a gradual process from the flocculent seeded sludge to compact aggregates, further to granular sludge, of 2.3 mm after three weeks of operation, and finally to mature granules of 3.5 mm of averaged diameter (day 60). The evolution of size distributions of the granules is shown for day 23, day 60 and day 220 in terms of number and volume percentage. Most of the volume percentage of the biomass on day 23 corresponded to granules with a size distribution between 1.2 and 3 mm (Figure 3.5A). However on day 220, the

volume percentage was shifted to diameters between 2.4 and 4.0 mm indicating the higher contribution of the big granules to the biomass concentration in the system.

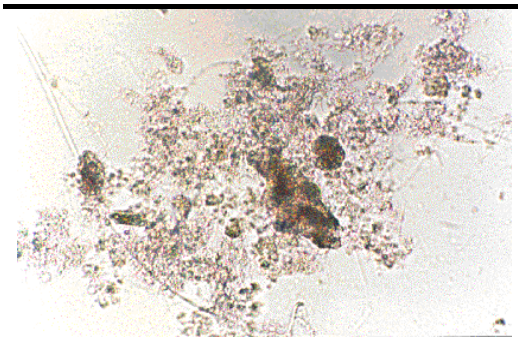
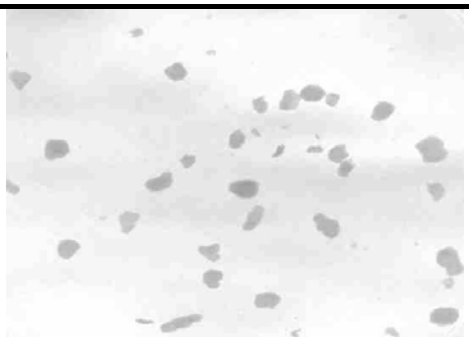
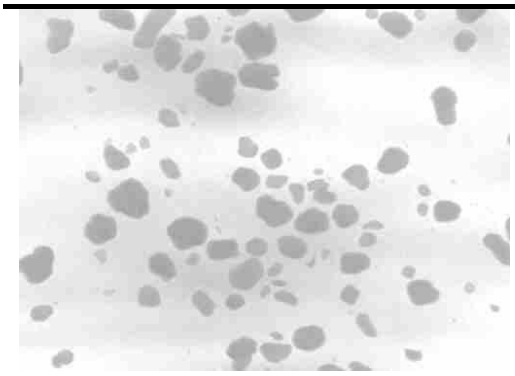
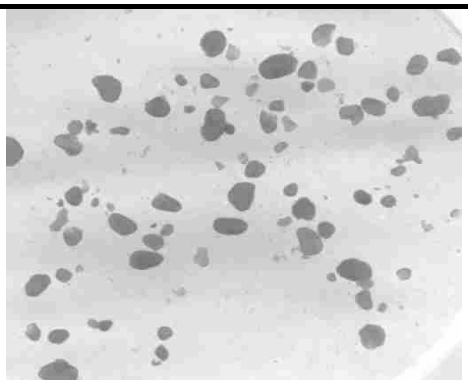
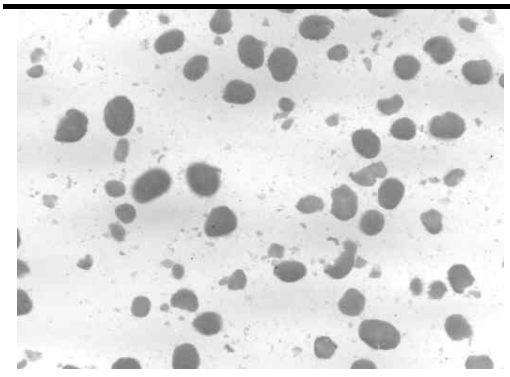
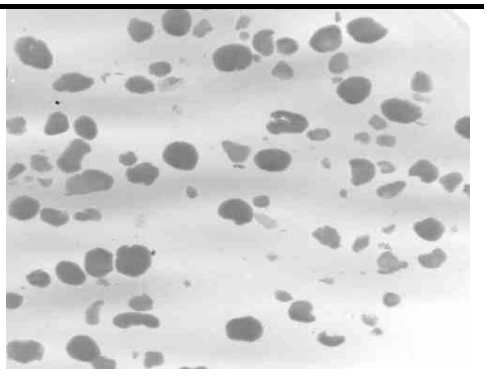
Operating day: 1 (A)	***Operating day :41***
	
Operating day:57	***Operating day: 170***
	
Operating day :259	***Operating day: 314***
	

Figure 3.4. Pictures of the seeding sludge (100x) (A) and the aerobic granules on different operating days (Reactor R1).

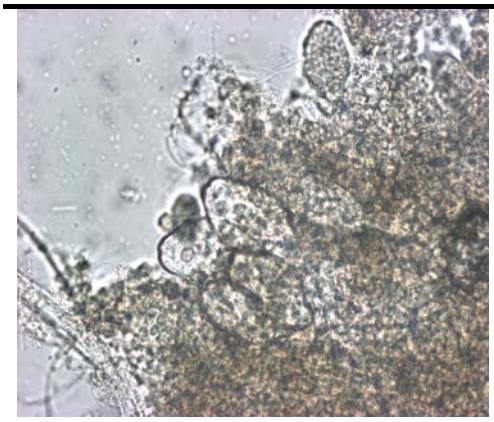
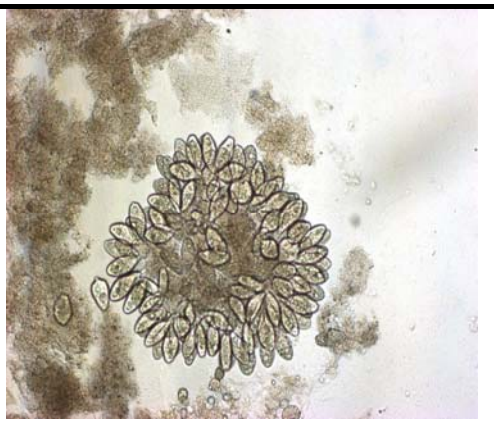
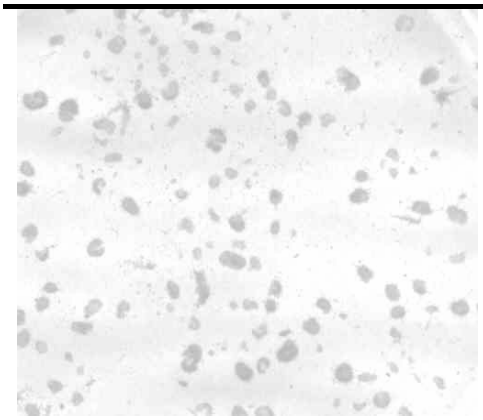
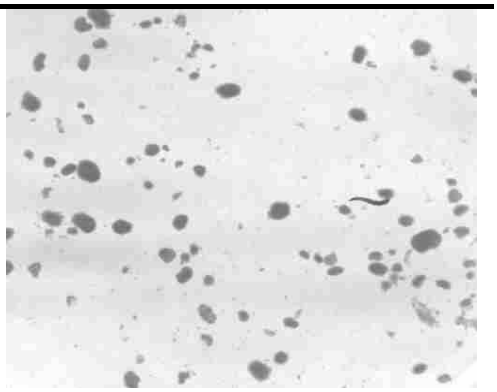
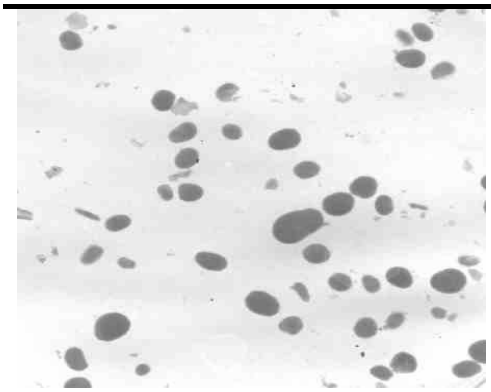
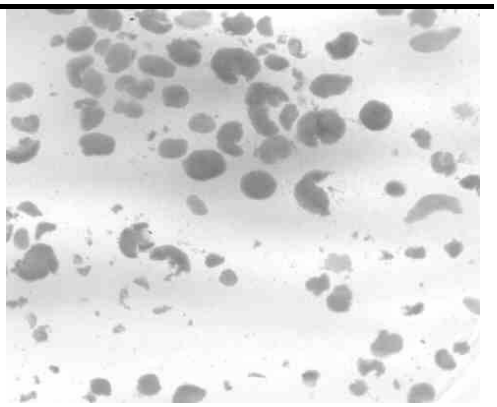
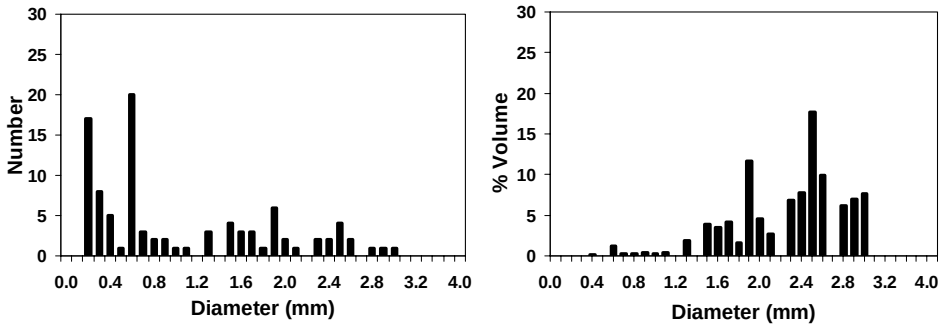
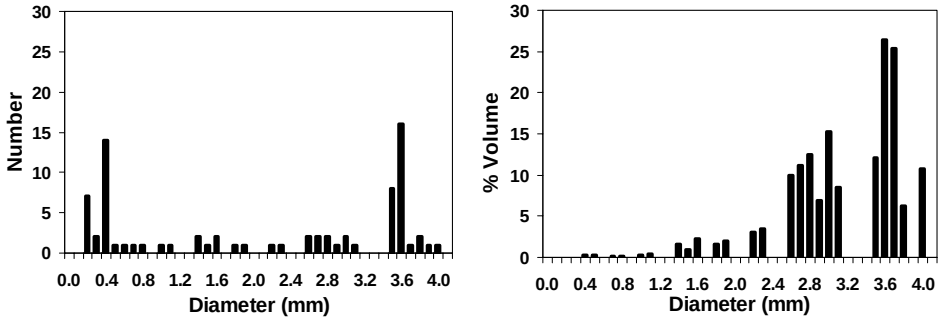
Operating day: 1 (A)	*Operating day :1 (A)*
	
Operating day:72	***Operating day: 170***
	
Operating day :219	***Operating day: 314***
	

Figure 3.5. Pictures of the seeding sludge (100x) (A) and the aerobic granules on different operating days (Reactor R2).

A) **Operating day 23**



B) **Operating day 60**



C) **Operating day 220**

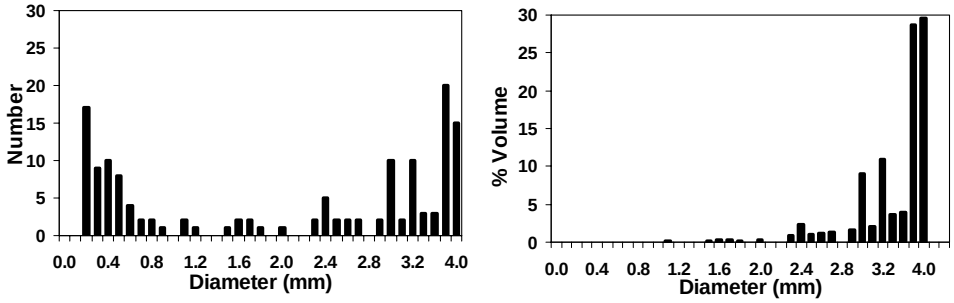


Figure 3.6. Comparison of the size distribution between granular sludge on operating days 23 (A), 60 (B) and day 220 (C) in R1.

During a first stage (first 219 operating days) the cycle distribution in both units was similar to the one used by Beun *et al.* (1999). Biomass concentration was around 0.2 g TSS/L at the beginning of the experiments (Figure 3.7). It increased up to 3 g TSS/L after 50 days, and then fluctuated at this level until it reached a stable value around 5-6 g TSS/L. The percentage of ashes of the solids from the reactors ranged from 5 to 13%. The biomass concentrations obtained during this study were similar to those obtained in the industrial SBR, between 3 and 9 g TSS/L, the VSS/TSS ratio being lower, from 0.6 to 0.8 g/g, although the biomass settling properties of the granules were much better. This made feasible to operate the granular reactors with an exchange volume of 50% because the volume occupied by the biomass was lower than 40 % of the reactor volume. Experimental results showed that the utilisation of an industrial wastewater apparently had no significant effect on the development of the granules and biomass accumulation in the system. During the first 50 days biomass accumulation was practically constant and estimated around of 44-54 mg VSS/d. Other authors obtained granules with similar physical characteristics to those obtained in this study, but they used synthetic media (Tay *et al*., 2002a; Etterer and Wilderer, 2001; Beun *et al*, 1999). On the other hand, Morgenroth (Morgenroth *et* al, 1997) obtained similar results using a molasses solution free of solids as feeding medium.

Biomass density was around 10-15 g VSS/(L-granules) in both reactors, which was similar to the value referred by Beun (Beun *et al*., 1999) of 11.9 g VSS/(L-granules). This value was lower than those reported for granules or biofilms formed in airlift reactors of 20-30 g VSS/(L-granules) (Kwok *et al*., 1996) and 15-20 g VSS/(L-granules) (Tijhuis *et al*., 1994). These authors found out that hydrodynamics and shear stress influenced strongly the biomass density, the higher the stress the higher the density of the biofilms. Furthermore, Villaseñor *et al*. (2000) postulated that the degree of reduction of the degraded carbon source or the maximum specific growth rate of the biomass on the substrate used affected the density of the obtained biofilm. They observed an important difference between the densities of biofilms developed with formate, 20-30 g VSS/(L-granules), formaldehyde, 25-35 g VSS/(L-granules), or methanol 100-120 g VSS/(L-granules). On the other hand, Tay *et al*. (2001c) indicated that

the granules obtained with different carbon sources, glucose or acetate, have comparable characteristics in terms of settling velocity, size, shape, biomass density and microbial activity. However, the microbial diversity of the granules was associated with the organic source supplied. In the present study the morphology of the granules obtained in both reactors fed with industrial wastewater were similar to those obtained with the synthetic feeding containing acetate as carbon source. Microbiological analysis to identify the microbial populations was performed by using samples of granules that were mechanically disrupted and analysed by the FISH technique. It was observed that nitrifying bacteria, corresponding to ammonia oxidizers, are present in both reactors due to the positive hybridization of the sampleswith the probe NEU635 indicating that they correspond to the genus *Nitrosomonas* (Figure 3.8).

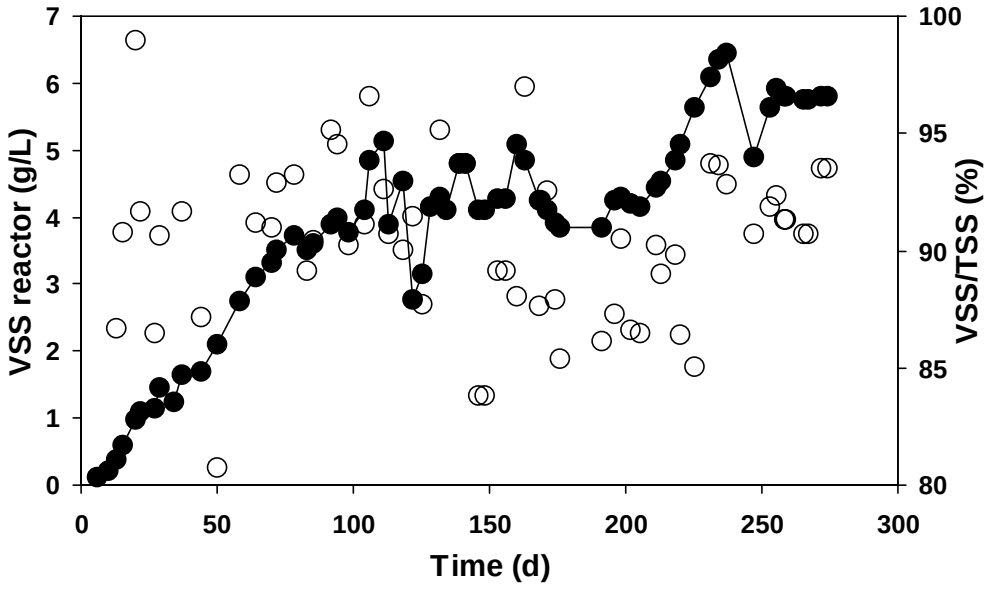
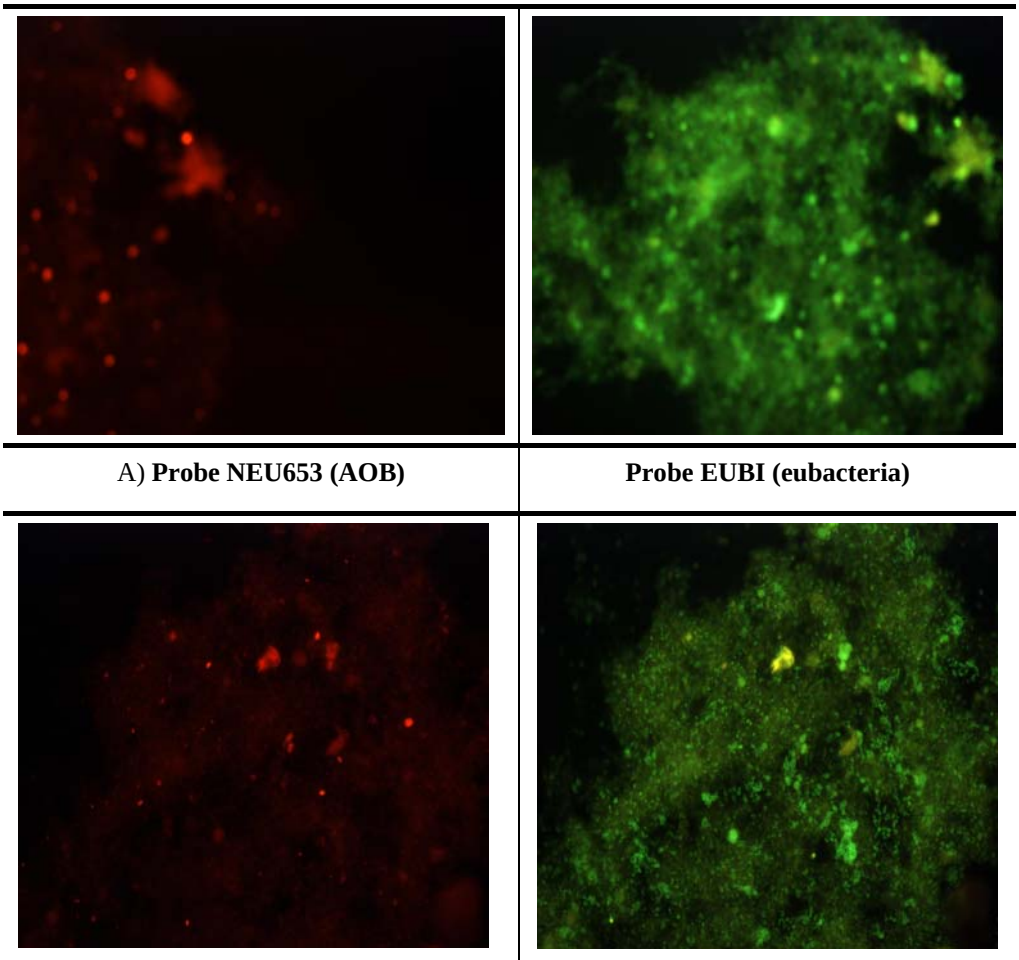


Figure 3.7. Concentration of TSS in the reactor R2 (•) and percentage of ashes in the TSS (O).



A) **Probe NEU653 (AOB)**	**Probe EUBI (eubacteria)**
B) **Probe NEU653 (AOB)**	**Probe EUBI (eubacteria)**

Figure 3.8. FISH in R1 (A) and R2 (B).

3.4.2. Carbon and nitrogen removal in the system

The reactor R1 was fed with acetate as sole carbon source for the first 27 operating days. During this stage the applied organic loading rate (OLR) and nitrogen loading rate (NLR) were of 1 g CODs/(L·d) and 0.1 g NH_4^+-N/(L·d), respectively. The main process for nitrogen removal in the SBR was the nitrogen assimilation for biomass growth and nitrification did not occur. During the following periods, the synthetic solution was progressively substituted by the raw

wastewater keeping the concentration of soluble COD constant of 1 g CODs/L and later the concentration was increased until the raw wastewater was fed without any dilution (Table 3.2). Nitrification of ammonia to nitrate was observed after one month of operation in both reactors. After the operating day 150, when the ammonia concentration was doubled due to changes on the composition of the wastewater, denitrification appeared simultaneously in both reactors. Nitrification and denitrification processes were the main mechanisms of nitrogen removal after this date. Nitrogen removal efficiencies were up to 80 % (on day 188) in both reactors. Applied OLR and NLR were comprehended between 1 and 7 g COD/(L·d) and between 0.1 and 0.7 g NH_4^+-N/(L·d), respectively in both reactors. (Figure 3.9; Figure 3.10).

The concentration of COD in the influent was between 0.5 and 1.8 g COD/L and the concentration of ammonia was between 30 and 180 mg NH_4^+-N/L. Overall COD removal efficiencies were, during the whole operational time, between 85 and 95%. Lower values than those and around 65 % were measured at occasional stages. The concentration of ammonia in the effluent ranged from 0 to 20 mg NH_4^+-N/L and nitrate concentration from 0 to 40 mg NO_3^--N/L (Figure 3.11). Simiral results were obtained in the case of R2 (Figure 3.12). These results are similar to those obtained by Garrido *et al*. (2001) treating the same industrial wastewater in an SBR of 28 m^3 that operated with flocculent sludge. However, in the granular SBR it was possible to operate at higher values of OLR and NLR, being the maximum values around five fold higher than in the industrial SBR and the obtained effluent with similar characteristics in terms of nitrogen and COD concentrations.

N removal efficiency was lower than 40% up to 160 operating day (Figure 3.13). From this day on, when the OLR and the NLR applied were increased, the nitrogen removal efficiency was also increased up to 85%.

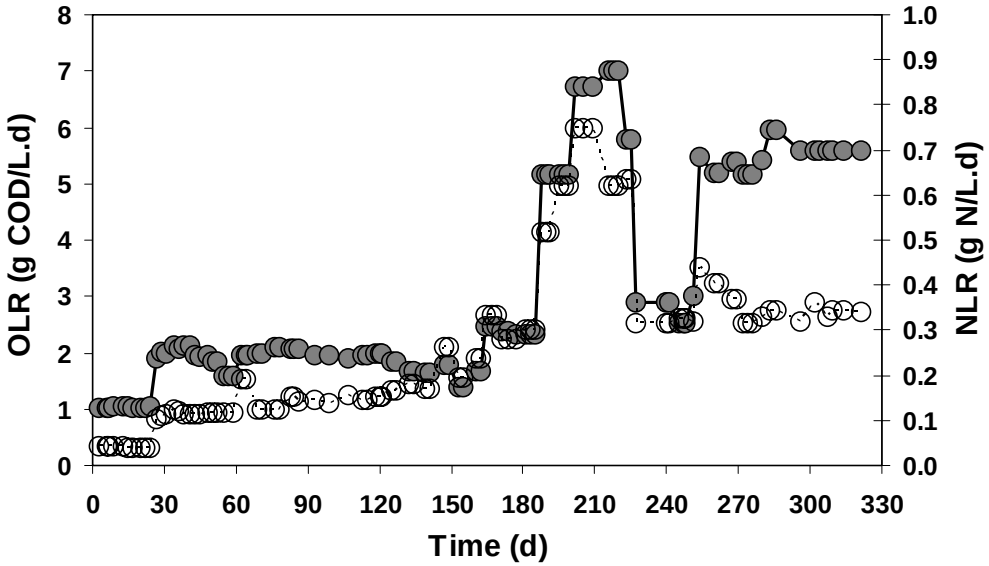


Figure 3.9. Organic loading rate (●) and nitrogen loading rate (○) fed to R1.

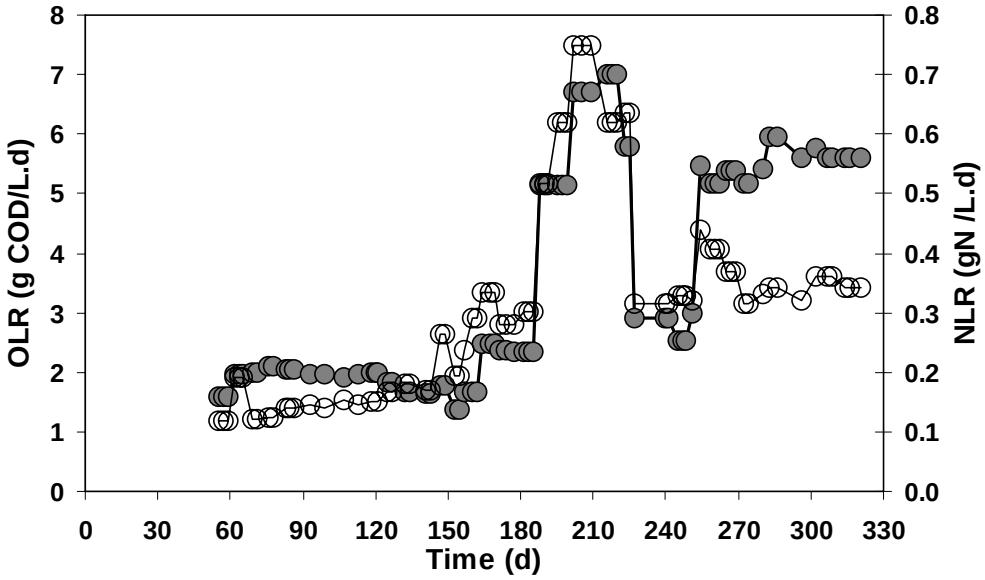


Figure 3.10. Organic loading rate (●) and nitrogen loading rate (○) fed to R2.

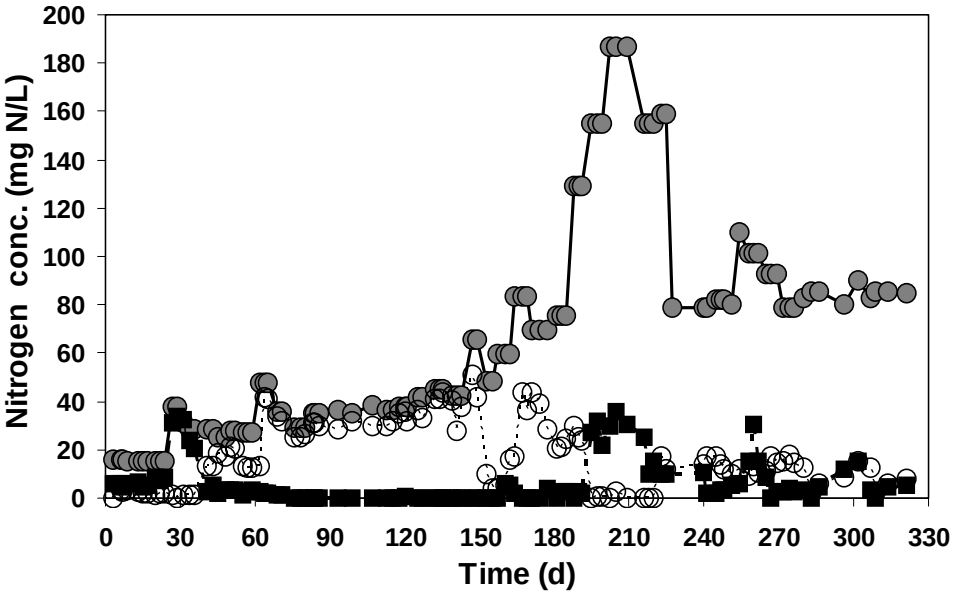


Figure 3.11. Ammonia concentration in the influent (●) and in the effluent (■), nitrate concentration in the effluent (O) from R1.

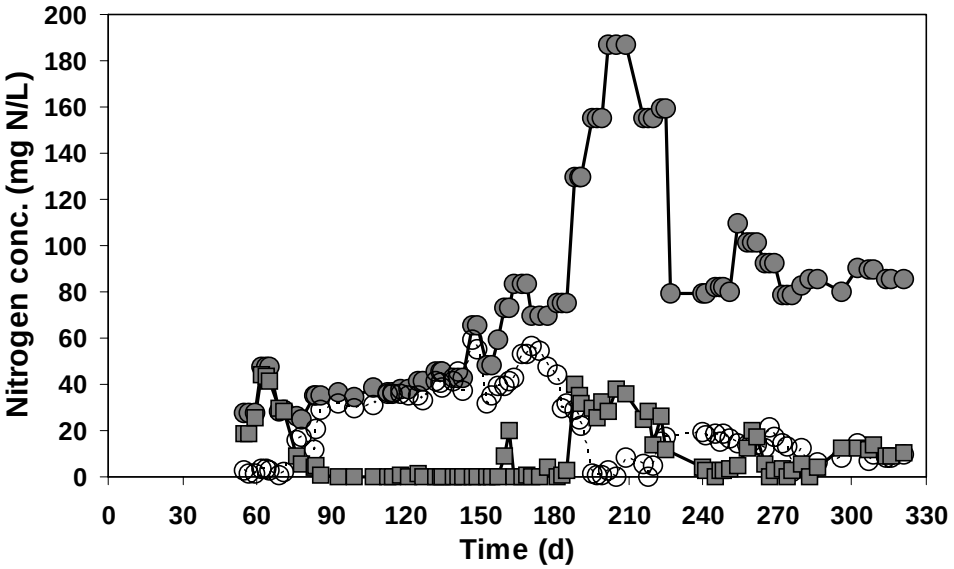


Figure 3.12. Ammonia concentration in the influent (●) and in the effluent (■), nitrate concentration in the effluent (O) from R2.

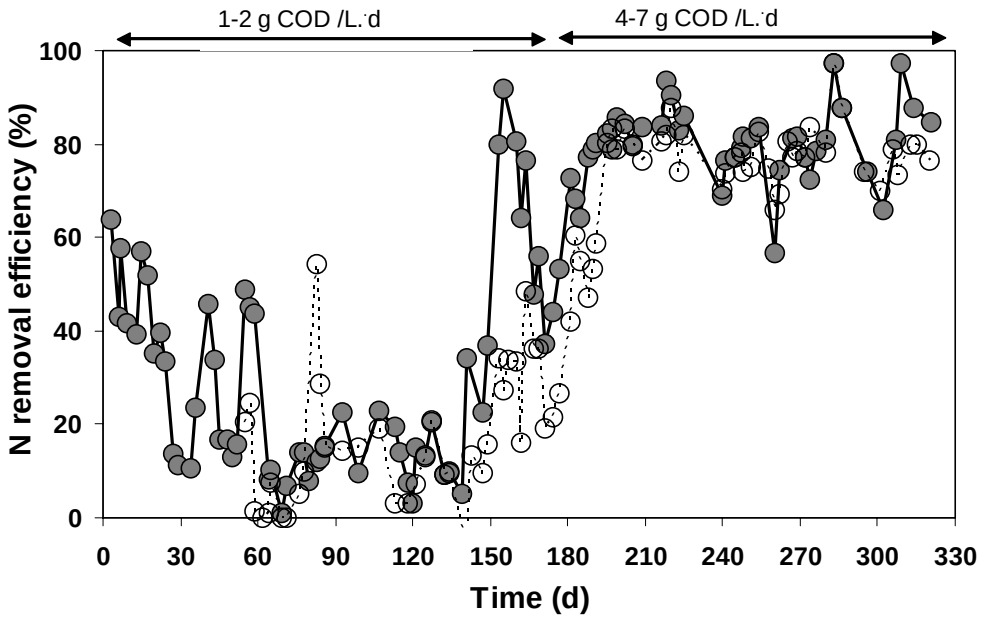


Figure 3.13. N removal efficiency in R1 (●) and R2 (O).

The measurement of the concentrations of these compounds was performed periodically along some operational cycles in order to explain the similar COD and nitrogen removal efficiencies obtained in both reactors at the end of each cycle. Typical concentration profiles during a cycle measurement are shown in the Figures 3.14 and 3.15 for the reactors R1 and R2, respectively.

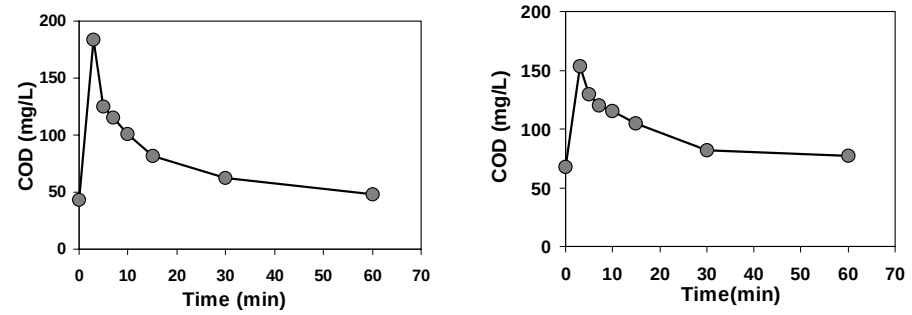
Figure 3.14 shows profiles measured on operating day 35 in R1 and on operating day 70 in R2. In both reactor, no nitrification or denitrification were carried out. However, Figure 3.15 shows profiles when both processes, nitrification and denitrification took place. These profiles were measured on operating day 260, when high OLR and NLR of 5 g COD/(L·d) and 0.4 g NH_4^+-N/(L·d), respectively, were applied. Except for the dissolved oxygen concentration (DO), the evolution of the concentrations of the measured compounds was similar in both reactors. The DO concentration in the R1 during the anoxic period was nearly 0.0 mg O_2/L and in the reactor R2 was of 3 mg O_2/L during first minutes of the cycle, and increased up to 6-7 mg O_2/L during the rest of the cycle. In case of the R1 during the aerobic period, DO concentration was near the saturation value of 7 mg O_2/L. Almost all the biodegradable COD and

nitrate concentrations disappeared completely in both cases during the first 10 minutes of the cycle (Figure 3.15 (R1)). The COD concentrations measured at the end of the cycle were similar in both systems and could be attributed to the fraction of slowly biodegradable substrate contained in the wastewater. Nitrate was consumed via denitrification while biodegradable compounds might be partly aerobically oxidized, partly used as electron donor for denitrification and partly stored in the biomass. Ammonia was oxidised to nitrate during the aerobic period immediately after the disappearance of biodegradable COD from the liquid phase.

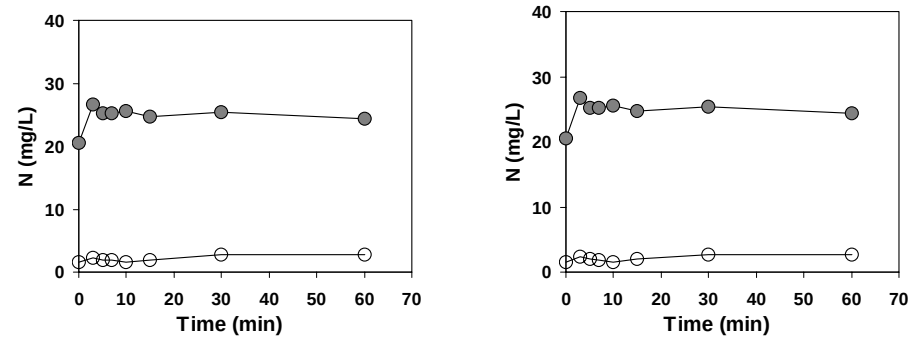
Due to the intrinsic characteristics of these SBRs, apparent denitrifying and nitrifying specific activities may be calculated from the cycle measurements as follows: the denitrifying activity from the nitrate consumption until depletion of this compound and the nitrifying activity from the slope of ammonia consumption after restoration of aeration (Mosquera-Corral *et al.*, 2005). The denitrification specific activity was of 0.28 g N/(g VSS·d) in R1, which is similar to 0.30 g N/(gVSS·d) obtained in the "aerobic" R2, but the nitrification specific activity was around 0.033 g N/(gVSS·d) in R2 and of 0.023 g N/(gVSS·d) in R1.

In case of reactor R2, the fact that the granules exhibited denitrifying activities under aerobic conditions may be explained because during the initial minutes of the cycle, a high amount of soluble biodegradable COD was fed, which increased drastically its concentration in the reactor. This caused a higher transport by diffusion of this organic matter fraction inside the granules than that of oxygen during this period. Moreover, the 3 mg O_2/L present during the feeding time of R2 were probably consumed by the outer layer of the granules and thus the inner layers, which were maintained under anoxic conditions, received enough carbon source and nitrate to support the denitrification process.

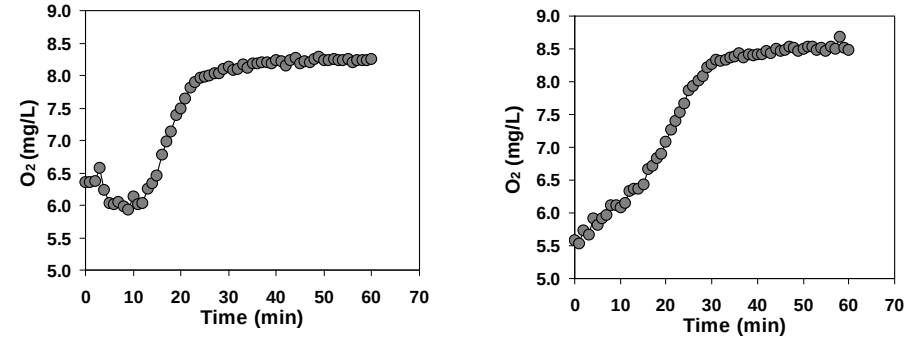
In these experiments, the ammonium loading rate was between 0.1 and 0.7 g NH_4^+-N/(L·d) and ammonium fed was nitrified, even when the organic loading rate fed was as high as 5-7 g COD/(L·d). These loading rates were higher that those referred by Dangcong *et al.* (1999) in other granular SBR, in which a high quality effluent was obtained using synthetic urban wastewater as feeding medium at 0.15-0.18 g NH_4^+-N/(L·d) and 1.5-2.0 g COD/(L·d).



A)



B)



C)

Figure 3.14. Typical concentration profiles during a cycle of the SBR in R1 on day 35 and R2 on day 70. A) (•) Total COD. B) (•) $N\text{-}NH_4^+$; (O) $N\text{-}NO_3^-$. C) Dissolved oxygen concentration.

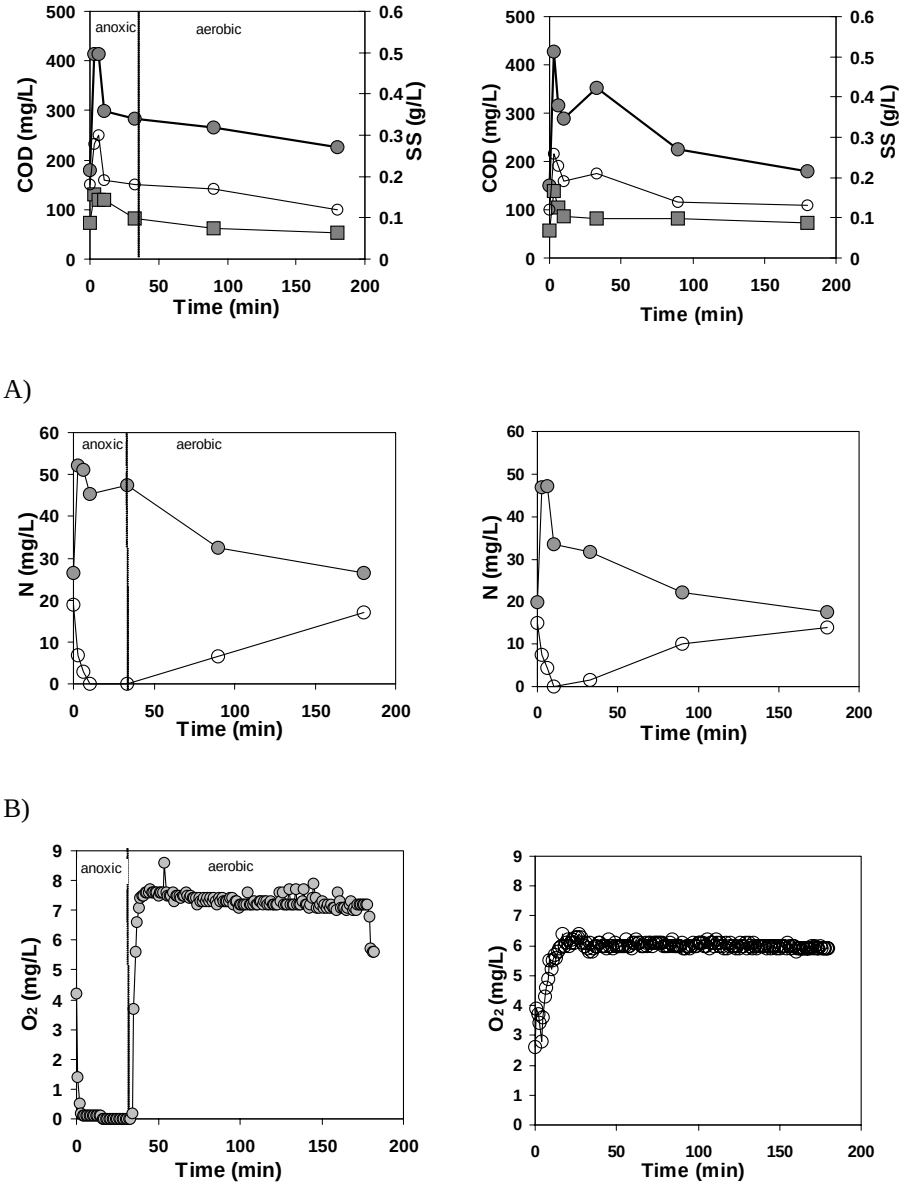


Figure 3.15. Typical concentration profiles during a cycle of the SBR in R1 and R2 on day 260. A) (•) Total COD; (O) TSS; (■) Soluble COD. B) (•) N-NH_4^+; (O) N-NO_3^-. C) Dissolved oxygen concentration.

3.4.3. Solids concentration in the effluent

An important parameter to take into account during the operation of the reactors in this study was the presence of suspended solids (TSS) in the industrial wastewater, which were not present in the composition of the influent of other granular SBRs (Tay *et al*., 2002a; Etterer and Widerer, 2001). The concentration of TSS in the industrial wastewater was high and ranged between 200 and 900 mg TSS/L, with a high fraction of volatile suspended solids (VSS) of around 75-97%. The amounts of suspended solids contained in the influent were partially removed in both SBRs, being the concentration of TSS in the effluent lower than in the influent, when the reactor was fed with industrial wastewater. The concentration of TSS in the effluent were comprehended between 50 mg TSS /L and 800 mg TSS/L for both reactors (Figure 3.16).

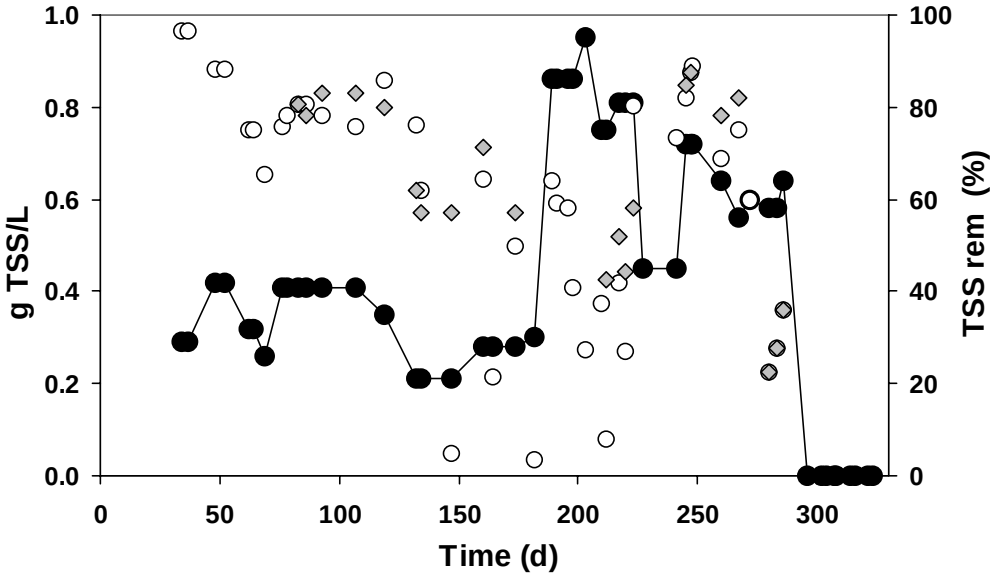


Figure 3.16. Concentration of TSS in the feeding (●) and TSS removal percentages in R1 (○) and R2 (◆).

The presence of TSS in the generated effluent is a major drawback for developing industrial scale granular SBRs. The advantages of granular SBRs, high conversion capacity and good settling properties of granules, are neutralized by the presence of suspended solids in the effluent. This is a paradox as granular

SBRs were usually presented in the literature as systems that may improve the behaviour of the bioreactor with regard to solids retention. The TSS in the effluent are constituted by the presence of very small flocs, which were always observed in the reactor. These small aggregates with relatively low ZSV are partly removed from the systems at the end of every operating cycle but a fraction of these aggregates remained in the systems.

The TSS removal percentage was defined by comparing the concentration of these in the influent and in the effluent, without considering a feasible generation in the reactors. The TSS removal percentages were in both reactors around 80 % with a decrease to lower values between days 150 and 220, which could be a result of the increase of OLR (Figures 3.9 and 3.16).

It was though that the capacity of the granular system to remove solids was related both with the daily amount of solids that were feed to the units and with the amount of biomass accumulated in the reactors. The relationship between the ratio of the CODp fed to the units and VSS in the reactors was plotted versus TSS in the effluent (Figure 3.17). Obtained results indicated that the TSS concentration in the effluent increased for CODp/SSV higher than 0.12 g/g. This indicated that it is necessary to limit the CODp/VSS ratio in the reactor below this value, in order to maintain a low TSS concentration in the effluent, as solids processing capacity of the granules is limited. A fraction of fed VSS can be degraded or accumulated in the system. Up to a concentration of 0.6 g VSS/L were removed between 90 and 220 days, however there was not relevant solids accumulation in the reactors after the first experimental weeks (Figure 3.7). Consequently, no significant accumulation of fed solids in the reactors took place, which indicated the degradation of the solids in the influent by the biomass.

A basic strategy for diminishing TSS in the effluent was tested. This strategy was based on the increase of the water withdrawal rate from the systems by diminishing the lenght of the withdrawal period. For this reason, on day 219, the effluent withdrawal time was manipulated from 3 to 1 min and on day 245 was diminished to 0.5 minutes. Afterwards it was sequentially increased from 0.5 to 1 min and from 1 min to 3 min (Table 3.2). With this strategy the partial retention of suspended solids previously observed decreased (Figure 3.18). Most of the

solids in suspension and small flocs were washed out during the first cycles after every reduction of the withdrawal time, and thus a new stable value for TSS in the effluent was achieved. In both systems average concentration of solids in the effluent was around 450 mg TSS/L, when the withdrawal time was 3 min, but it decreased strongly to 200 and 150 mg TSS/L when the withdrawal time was diminished to 1 and 0.5 min, respectively. This proved that the strategy had a positive effect in the effluent quality regarding to solids concentration.

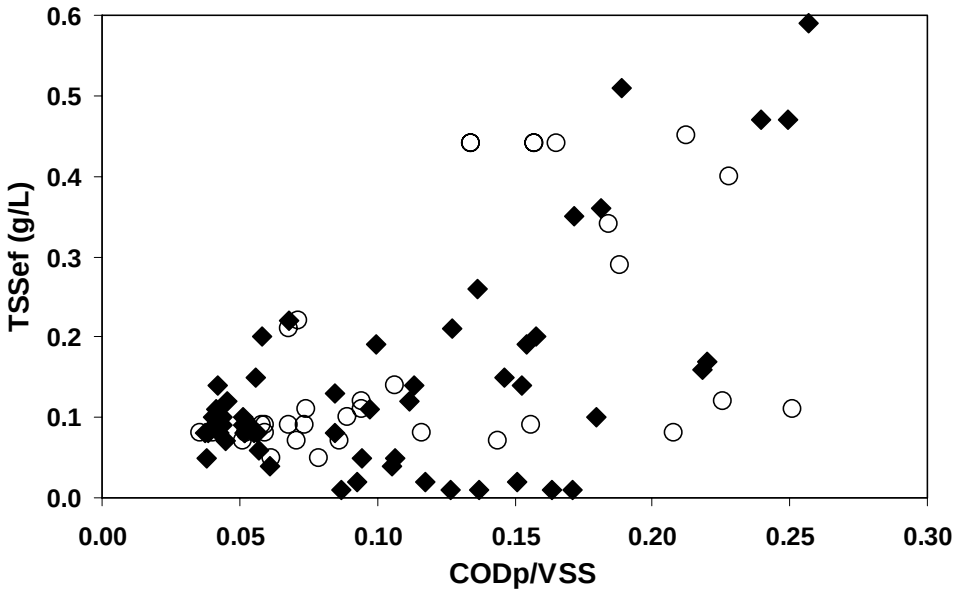


Figure 3.17. Evolution of the CODp/ fed-VSS on the TSS concentration in the effluent of R1 (♦) and R2 (○). Data obtained from the experiments carried out with industrial wastewater as feeding medium and length of the withdrawal period 3 min.

From day 296 both reactors were fed with the same free of solids synthetic wastewater which was used during the start up of R1. The concentration of suspended solids in the effluent was around 200 mg TSS/L, which were lower than the 450 mg TSS/L that were measured using the industrial wastewater with the same withdrawal time of 3 min. This indicated that the solids present in the effluent not only depended on the biomass washed out from the reactor, but also on the solids content of the feeding. On the other hand, Figure 3.18 showed that the content of TSS in the effluent during this experiment was similar or even

higher than the value obtained when the industrial wastewater was fed to the system with a withdrawal time of 1 min or 0.5 min. The presence of biomass in the effluent, even when no solids are present in the feeding media, might be originated by the breaking up of the granules in fragments caused by diffusion limitation of substrates and subsequent cellular decays by lysis. This fact occurred when the size of granules was too big and a decay processes might then take place in the inner zone of the granules as it occurs in the case of biofilms (van Benthum *et al*., 1996). From an experiment with a nitrifying lab-scale biofilm airlift suspension (BAS) reactor (van Benthum *et al*., 1996) lasting more than 500 days, the continuous break-up of granules was observed. In the effluent of the BAS reactors small fragments, size 0.2 to 0.3 mm, were always observed, broken off from larger granules or biofilms and washed out.

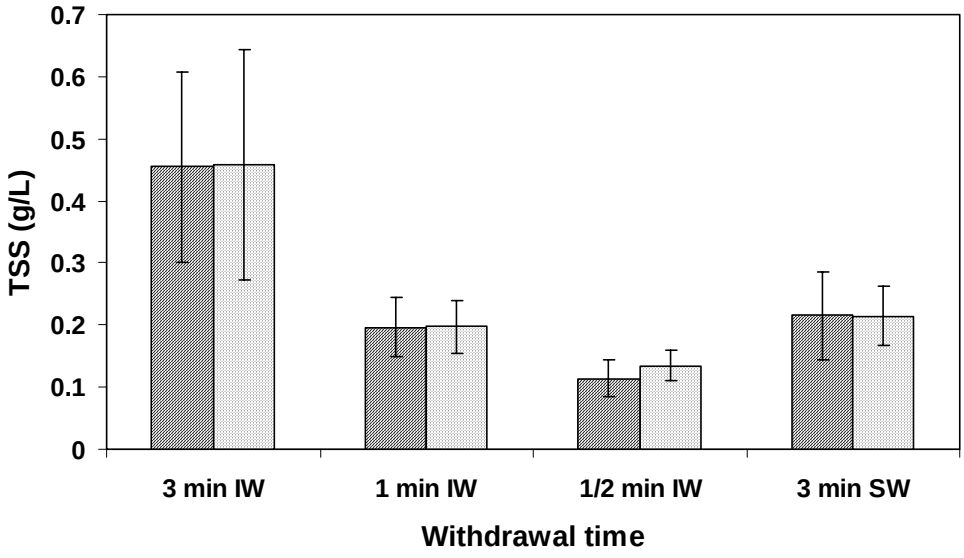


Figure 3.18. Evolution of the effluent TSS on the water withdrawal time (OLR between 5 and 7 g COD/L.d). IW = industrial wastewater and SW = synthetic wastewater without TSS.

Experiments shown that there are at least 3 causes that originated the presence of small aggregates in the SBR reactors: the own presence of TSS in the influent, the generation of small biomass aggregates during the cycle due to biomass growth and the small biomass patches that are detached from the granules. The first and last causes were pointed out during our studies. The

second cause, growth of small biomass aggregates was demonstrated during experiments in which withdrawal time was manipulated, as the modification of this parameters do not appear to influence either detachment or the retention of the feeding solids by the granules.

3.5. Conclusions

- The formation of granules in two SBRs was achieved by using a industrial wastewater coming from a dairy analysis laboratory as influent. Granules with good settling properties were obtained, SVI of 60 mL/g VSS, and ZSV of 20 m/h. This made feasible to operate the system with high exchange volume and thus organic and nitrogen loading rates applied to both systems were high, up to 7 g COD/(L·d) and 0.7 g NH_4^+-N/(L·d).
- Nitrogen removal efficiency was similar in both units, even considering that R2 was operated always under aerobic conditions. Nitrogen and COD removal efficiencies were 80 and 70 %, respectively. Nitrate disappeared in both units during the first minutes of the feeding period, although in R2 dissolved oxygen concentration was higher than 3 mg O_2/L. Thus, denitrification might take place in R2 in the inner core of the granules, oxygen being depleted by the outer layers.
- The TSS concentration in the effluent was between 50 and 700 mg TSS/L during the different operational stages. It was found that the presence of TSS in the effluent was a result of at least of three causes: the own presence of TSS in the influent; the detachment of small biomass patches from the granules and the growth of small flocs that were washed out.
- The presence of TSS in the effluent of the SBRs was strongly affected by either the length of the withdrawal period or by the applied particulated COD to biomass ratio (CODp/VSS) to the systems.
    - The TSS in the effluent, lower than 200 mg TSS/L, were attained when the systems operated with an CODp/VSS ratio lower than 0.12 g COD/g VSS.

- With regard to the effect of the length of the withdrawal period, there was a strong reduction of the averaged TSS content in the effluent from 450 to 200 and 150 mg TSS/L when this time was diminished sequentially from 3 to 1 and 0.5 min, respectively. This was caused by a more intensive washout of small suspended biomass aggregates that took place when the lenght of this period was shortened, which reduced proliferation of small flocs in the systems. Anyway, results pointed out that the presence of solids in the effluent of granular SBRs is intrinsic with the behaviour of these units. To resume, diminution of withdrawal time and the limitation of CODp/VSS applied to the systems have a positive effect on the TSS in the effluent.

3.6. References

Amann R.I. (1995). *In situ* identification of micro-organisms by whole-cell hybreidization with r RNA-targeted nucleic acid probes, p.1-15. In A.D.L: Akkerman, J.D. van Elsas, and F.J. de Brujin (ed.), *Molecular microbial ecology manual*. Kluwer Academic Publisher, Dordrecht, The Netherlands.

APHA-AWWA-WPCF. (1999). Standard Methods for the examination of water and wastewater. 20th Edition. Clesceri, L.S., Greenberg, A.E. & Eaton, A.D. (eds.).

Arrojo B**.**, Omil F., Garrido J.M. and Méndez R. (2003). Combinación de un filtro anaerobio y un sistema SBR para el tratamiento de las aguas generadas en un laboratorio de análisis de productos lácteos. *Afinidad,* **60**, 344-354.

Blackall L.L., Harbers A. E., Greenfield P.F. and Hayward A.C. (1991). Foaming in activated sludge plants: a survey in Queensland, Australia and an evaluation of some control strategies. Water Research, **25** (3), 313-317.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P:A: and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research,* **33** (10), 2283-2290.

Dangcong P., Bernet N., Delgenes J-P and Moletta R. (1999). Aerobic granular sludge- A case report. *Water Research* **33,** (3), 890-893.

Etterer T. and Wilderer P.A. (2001). Generation and properties of aerobic granular sludge. *Water Science and Technology,* **43** (3), 19-26.

Fernández-Carrasco E. Omil F., Garrido J.M., Arrojo B**.** and Méndez R. (2004). Advanced monitoring and supervision of biological treatment of complex dairy effluents in a full-scale plant. *Biotechnology Progress,* **20,** 992-997

Garrido J. M., Omil F., Arrojo B., Méndez R.. and Lema J. M**.** (2001). Carbon and nitrogen removal from a wastewater of an industrial dairy laboratory with a coupled anaerobic filter-sequencing batch reactor system. *Water Science and Technology,* **43**, 315–321.

Hailei W., Yu G., Liu G. and Feng P. (2006). A new way to cultivate aerobic granules in the process of papermaking wastewater treatment. *Biochemical Engineering Journal* (2006), **28**(1), 99-103.

Jeison D. and Chamy R. (1998). Novel technique for measuring the size distribution of granules from anaerobic reactors for wastewater treatment. *Biotechnology Techniques,* **12** (9), 659-662.

Jiménez B., Noyola A., Capdeville V., Roustan M. and Faup G. (1988) Dextran Blue coulorant as a reliable tracer in submerged filters. *Water Research,* **22**, 1253-1257.

Kwok W.K., van Loosdrecht, M.C. M. and Heijnen J. J. (1996). Application of a biofilm airlift suspension reactor for acetic acid removal. Internal report, Delft University of Technology, Delft, The Netherlands.

Lettinga G., Hulshoff Pol L.W. and Zeeman, G. (1993). Biological Watewater Treatment, Anaerobic Treatment. Department of Environmental Technology, Wageningen Agricultural University, The Netherlands.

Liu Y., Xu H.L., Yang S.F. and Tay J.H. (2003). Mechanisms and models for anaerobic in upflow anaerobic sludge blanket reactor. *Water Research,* **37**, 661-673.

Liu Y. and Liu, Q.-S. (2006). Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. *Biotechnology Advances,* **24**(1), 115-127.

Morgenroth E., Sherden T., Van Loosdrecht M.C.M., Heijnen J.J. and Wilderer P.A. (1997). Aerobic granular sludge in a sequencing batch reactor. *Water Research*, **31** (12), 3191-3194.

Mosquera-Corral A., de Kreuk M.K., Heijnen J.J. and van Loosdrecht M.C.M. (2005). Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. *Water Research*, **39** (12), 2676-2686.

Ng Y.H., Say L., Ong L. and Ng W.J. (2005). Effects of sodium chloride on the performance of a sequencing batch reactor. *Journal of Environmental Engineering*, **131** (11), 1557-1564.

Omil F., Garrido J.M., Arrojo B. and Méndez R. (2003). Anaerobic filter reactor performance during the treatment of complex dairy wastewaters at industrial scale. *Water Research*, **37**, 4099-4108.

Pujol R., Duchene Ph., Schietrite S. And Canler J.P. (1991). Biological foams in activated sludge plants: characterization and situation. *Water Research*, **25** (11), 1399-1404.

Smolders G. J. F., Klop J., van Loosdrecht M.C. M. and Heijnen J.J. (1995). A metabolic model of the biological phosphorus removal process. Effect of the sludge retention time. *Biotechnology and Bioeng*ineering, **48**, 222-233.

Soto M., Veiga M.C., Méndez R. and Lema J.M. (1989). Semi-micro COD determination method for high salinity wastewater. *Environmental Technology Letters*, **10**(5), 541-548.

Tay J-H, Liu Q-S and Liu Y. (2001a). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology*, **33**, 222-226.

Tay J-H, Liu Q-S and Liu Y. (2001b). The effects of shear force on the formation, structure and metabolism of aerobic granules. *Applied Microbiology and Biotechnology*, **57**, 227-233.

Tay J-H, Liu Q-S and Liu Y. (2001c). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *Journal of Applied Microbiology*, **91**, 168-175.

Tay J-H, Liu Q-S and Liu Y. (2002a). Aerobic granulation in sequential sludge blanket reactor. *Water Science and Technology*, **46** (4-5), 13-18.

Tay J-H, Liu Q-S and Liu Y. (2002b). Hydraulic slection pressure-induced nitrifying granulation in sequencing batch reactors. *Applied Microbiology Biotechnology*, **59**, 332-337.

Tijhuis L., van Loosdrecht M.C.M. and Heijenen J.J.(1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioeng*ineering, **44**, 595-608.

van Benthum (1996). Integrated nitrification and denitrification in biofilm airlift reactors: Biofilm development, process design and hydrodynamics. PhD Thesis, Technical University Delft, The Netherlands.

Villaseñor J.C., van Loosdrecht M.C.M., Picioreanu C. and Heijnen J.J. (2000). Influence of different substrates on the formation of biofilms in a biofilm airlift suspension reactor. *Water Science and Technology*, **41** (4-5), 323-330.

Wang Z.-W., Liu Y. and Tay J.-H. (2006). The role of SBR mixed liquor volume exchange ratio in aerobic granulation. *Chemosphere*, **62**(5), 767-771.

Wanner J., Ruzickova I., Jetmarová P. Krhutkova O. and Paraniaková J. (1998). A national survey of activated sludge separation problems in the czech republic: filaments, floc characteristics and activated sludge metabolic properties. *Water Science and Technology*, **37** (4-5), 271-279.

Chapter 4

Nitrifying granular sludge production in a SBR[1]

Summary

The first objective of this research was to obtain a nitrifying granular sludge from heterotrophic sludge by decreasing the COD/N until COD was completely eliminated. In spite of the changes in the feeding composition the granules maintained their structures and the solids content in the effluent was reduced to 10 mg TSS/L when acetate was removed from the feeding media.

The second objective was to study the effect of different carbon to nitrogen ratios (COD/N) in the feeding on the production of nitrogen compounds in the effluent. The feeding flow was a synthetic medium, which contained acetate as carbon source (1-0 g COD/L) and ammonium as N source (30-200 mg NH_4^+-N/L). Different COD/N ratios of 15, 7, 5, 2.5, 1.25 and 0 g/g in the feeding were tested. The COD removal percentage was around 90% during the whole operational period. Changes on the COD/N ratio provoked the presence of different concentrations of nitrogen compounds in the effluent. The N removal percentages obtained in the reactor were up to 55%. Removal of ammonia was carried out by both assimilation and simultaneous nitrification-denitrification processes. The predominance of each mechanism was related to the COD/N ratio in the feeding media.

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[1] **Mosquera-Corral A., Vázquez-Padín J.R., Arrojo B., Campos J.L. and Méndez R.** (2005). Nitrifying granular sludge in a Sequencing Batch Reactor. *Aerobic Granular Sludge. Water and Environmental Series IWA Publishing*, 63-70.

4.1. Introduction

Activated sludge is one of the most common biological processes used for wastewater treatment of organic and nitrogenous compounds. Organic matter is normally removed by aerobic oxidation and nitrogen compounds by the combination of two processes: the aerobic nitrification and the anoxic denitrification. Usually, in these units, the loading rate is limited by the maximal biomass concentration that can be maintained inside the system. To maintain high biomass concentrations inside the system, settlers with very large areas are required. Besides the nitrification process is the limiting step for the treatment of nitrogenous compounds due to the slow growing rate of the microorganisms involved. This fact provokes the necessity of achieving a high solid retention time (SRT) and, therefore, a large volume of the system.

One option to increase the sludge retention time (SRT) consists of the immobilization of microorganisms in form of biofilms or granules. High biomass concentrations and low unit volumes are achieved with biomass immobilization either on the surface of a support material or by autoaggregation in form of granules. The biofilm airlift suspension (BAS) reactor is an example of this kind of reactors where the biomass grows adhered on an inorganic support. Many research works have been focussed on the study of these reactors (Tijhuis *et al*., 1994; van Benthum *et al*., 1997). The biomass grown in biofilms, in continuously operated reactors, can accomplish different processes at the same time. Recently aerobic granular sludge was developed in a sequencing batch airlift reactor (SBAR) (Beun *et al*., 1999) where the removal of COD as well as nitrification and denitrification processes occurred (Beun *et al*., 2001).

In general, the population distribution in biofilms is the result of the difference in growth rates. The slow-growing nitrifiers are located inside the biofilms, the fast-growing heterotrophs are located more in the outer layer of the biofilms (van Loosdrecht *et al*., 1995). In a discontinuously fed system, the organic carbon source penetrates completely into the biofilms because of the temporarily high concentration in the liquid. Dissolved oxygen is present only in the outer layer of the biofilms due to its consumption rate. Consequently, it can be expected that the nitrifiers are located in the outer aerobic layer of the biofilms

(de Beer *et al*., 1993) together with the heterotrophs oxidizing aerobically the organic matter, and the organic carbon compounds are stored anoxically as PHB (Poly Hydroxy Butirate) by the heterotrophs inside the biofilms. In the inner core of the granule the heterotrophs grow using organic matter and reducing nitrate generated by nitrification to nitrogen gas. They were also present in the outer layers of the granules where they could use oxygen as electron acceptor.

Nitrogen removal from wastewater via biological processes of nitrification-denitrificatin is dependent on the COD/N ratio of the watewater treated (Sánchez *et al*., 2000; Komorowska-Kaufman *et al*., 2006). The effects of different carbon substrates (glucose, acetate, methanol, wastewater), and the C/N ratio, on the denitrification process have been studied (Tam *et al*., 1992; Akunna *et al*., 1993). It has been observed that the use of acetic acid as the carbon source ensures high denitrification rates (Carley and Mavinic, 1991; Mateju *et al*., 1992). Different intermediates (NO_2^-, N_2O, NO) may accumulate during denitrification, depending on the kind and concentration of substrate, operational conditions, the presence of toxic substances and the competition between microorganisms (Knowles, 1982; Polprasert and Park, 1986). Therefore, it is necessary to control operational conditions to ensure a proper denitrification.

The effect of the COD/N ratio on the nitrification and denitrification processes using flocculent sludge has been widely studied elsewhere (Buys *et al*., 2000). Knowledge of the specific denitrifying activity (SDA) of sludge makes it possible to calculate the maximum nitrogen load that may be treated by a system. Sánchez *et al.* (2000) evaluated the effects of C/N ratio on the determination of the SDA to establish the optimum operational conditions.

Nutrient removal has been demonstrated in aerobic granular SBRs, including nitrification (Tay *et al*., 2002; Tsuneda *et al*., 2003; Tokutomi, 2004), N removal under alternating aerobic/anaerobic conditions (Jang *et al*., 2003; Yang et al., 2003), simultaneous nitrification and denitrification (Beun *et al*., 2001, 2002; Cassidy and Belia, 2005) an biological P removal (Lin *et al*., 2003). Tsuneda *et al.* (2006) studied the nitrification with nitrifying granules applied various types of watewaters such as inorganic wastewater containing a low concentrations of saline and ammonia, and domestic wastewater containing both organic pollutants

and ammonia. These authors also evaluated the performance of the nitrifying granules on the basis of the maximun nitrification rate, ammonia removal efficiency and stability through the long-term operation.

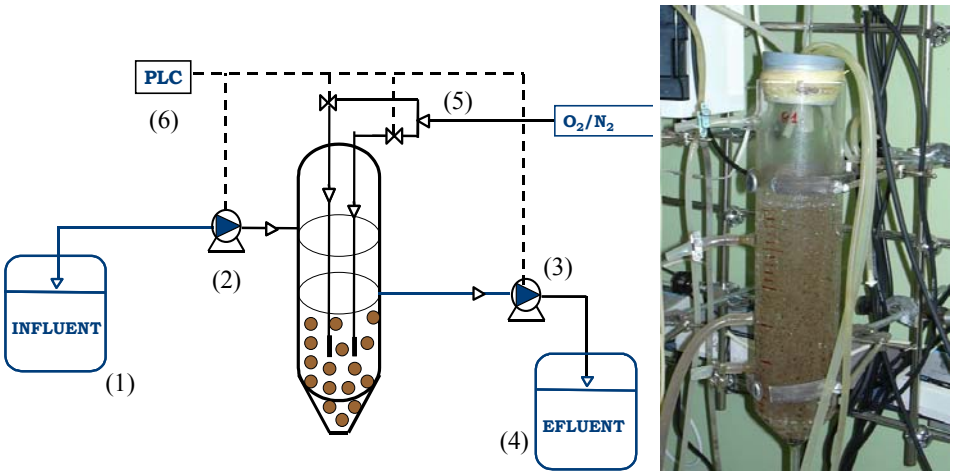
4.2. Objectives

- To study the effect of the COD/N ratio in the feeding on the removal efficiency of organic matter and nitrogen compounds in a granular SBR
- To study the formation and operation of nitrifying granular biomass in a granular SBR.

4.3. Materials and methods

4.3.1. Experimental set-up

A sequencing batch reactor (SBR) with a total volume of 2.5 L and a working volume of 1.5 L was used. Dimensions of the unit were: height of 465 mm and inner diameter of 85 mm, the height to the diameter ratio (H/D) being 5.5. The maximum level of the liquid was 264 mm, and the minimum level 132 mm after effluent withdrawal. Oxygen was supplied by using spargers to promote the formation of small air bubbles. A set of two peristaltic pumps was used to feed and to discharge the effluent, respectively. The influent was introduced in the system through ports located at the top of the reactors. The effluent was discharged through the sampling port placed at middle height of the column reactor (Figure 4.1). A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations over the pumps and valves, and thus the length of every operational period in the SBR. The reactor was operated at room temperature (15-20 ºC), at oxygen concentrations of 6-8 mg O_2/L (during the feast period) and 8 mg O_2/L (during the famine period) and without pH control, which varied between 7.4 and 8.5.



A) B)

Figure 4.1. A) Experimental set up: (1) Feeding tank; (2) Feeding pump; (3) Effluent pump; (4) Effluent tank (5); Air valve; (6) PLC. B) Picture of the reactor.

4.3.2. Inocula

The granular SBR was previously operated performing the experiments presented in Chapter 3 and corresponded to R2.

4.3.3. Feeding media

The SBR was fed with a synthetic solution with the composition described in Table 4.1 according to Beun *et al*. (1999) and the trace solution to Smolders *et al*. (1995). The system was inoculated with a granular sludge collected from another SBR.

Table 4.1. Composition of the synthetic wastewater used to feed the SBR.

Parameters	Concentration (mg/L)
COD	0-1000
NH_4^+-N	30-200
PO_4^{3-}-P	23
Traces	1 mL/L

4.3.4. Strategy of operation

The operational conditions of the reactor are summarized in Table 4.2. Different COD/N ratios of 15.0, 7.0, 5.0, 2.5, 1.25 and 0 g/g in the feeding were used. The system was operated in cycles of 3 hours with an exchange volume of 50% (Figure 4.2). The hydraulic retention time (HRT) was always 0.25 days.

Table 4.2. Averaged values during the different operational stages of the SBR.

Period	Days (d)	COD (mg/L)	NH_4^+-N (mg/L)	COD/N (g/g)
I	12-70	500	100	5.0
II	71-160	500	200	2.5
III *	161-203	500	200	2.5
IV	204-210	500	33	15.0
V	211-246	500	72	7.0
VI	247-322	62.5	50	1.25
VII	323-342	0	50	0
VIII	343-430	0	100	0

* Removal of 5.25 g VSS from the reactor.

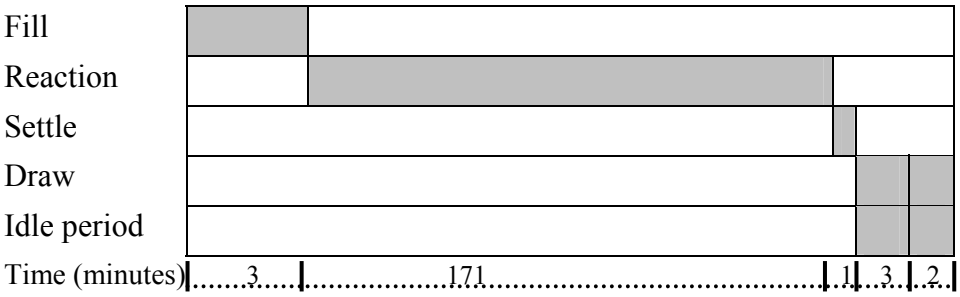


Figure 4.2. Operation strategy of the SBR during a cycle.

4.3.5. Analytical methods

The pH, nitrate, ammonia, TOC, volatile suspended solids (VSS), total suspended solids (TSS) and SVI were determined according to Standard Methods (APHA, 1999) as described in Chapter 2. Concentrations of chemical oxygen demand (COD) were determined by a modified method from Standard Methods (Soto *et al*., 1989).

Biomass density, in terms of biomass per volume occupied by the granules (g VSS/ ($L_{granules}$)), was determined using dextran blue, which is not absorbed by the biomass (Jiménez *et al*., 1988) and following the methodology proposed by Beun *et al*. (1999). Biomass was observed using a Stereomicroscope ZMZ-2T (Nikon) combined with a digital camera (Coolsnap, Roper Scientific Photometrics) (Section 2.3 of Chapter 2). Morphological studies of the biomass were performed with a scan electron microscope (Digital SEM Leica 440 at 20 kV) controlled with a computer system and with a magnification capacity ranging from 15 to 290000 folds. The sludge sample was washed with phosphate buffer and subsequently fixed with a solution of glutaraldehyde and then the samples were dehydrated using ethanol (Section 2.3 of Chapter 2).

The presence of nitrifying bacteria in granules was followed by Fluorescence *in situ* hybridization (FISH) (Section 2.4 of Chapter 2).

4.3.6. Calculations

For each period the amount of biomass produced was estimated from the biomass increase in the reactor and the amount of biomass washed out in the effluent using equation [4.1]:

$$DW_P = \Delta Xr \cdot Vr + \bar{X}e \cdot Q \cdot \Delta t \qquad \text{(g VSS)} \qquad [4.1]$$

Where: DW_P = amount of produced biomass (g VSS), ΔXr = change of biomass concentration during each period (g VSS/L), Vr = reactor volume (L), $\bar{X}e$ = averaged biomass concentration washed out in the effluent (g VSS/L), Q = flow rate (L/d) and Δt = length of each period (d). Considering a general composition of the biomass as $C_5H_7NO_2$ the averaged amount of nitrogen assimilated for biomass growth (DW_N) was calculated using equation [4.2] as:

$$DW_N = DW_P \cdot \frac{14 \text{ g-mol N}}{113 \text{ g-mol Biomass}} \qquad (\text{g N}) \qquad [4.2]$$

Dividing the averaged amount of the nitrogen present in the produced biomass (DW_N) by the flow rate (Q) and the duration of each period (Δt) and referred to the inlet ammonia concentration, the percentage of nitrogen assimilated is calculated. N_R and N_A were calculated related to the nitrogen amount in the feeding. Nitrogen removed by denitrification (N_D) was calculated by the difference between both of them.

4.4. Results and discussion

The granular SBR was operated in eight different stages fed with a synthetic media.

4.4.1. Nitrogen removal

During Period I the reactor was operated with a COD/N ratio of 5 g/g during 58 days. The ammonia concentration in the influent was 100 mg N/L and the NLR of 0.4 g N/L·d (Figure 4.3). After 30 days of operation stable conditions were reached and the percentage of nitrogen removal was 48-54%. At this point the concentrations of inorganic nitrogen compounds in the effluent were around 12 mg NO_3^--N/L and 17 mg NO_2^--N /L. During the next 90 days the COD/N ratio applied was 2.5 g/g (Period II). The ammonia concentration in the influent was increased to 200 mg N/L and after the first 30 days stable conditions were reached and percentage of nitrogen removal was 33-41%. Concentrations in the effluent were around 30 mg NO_3^--N/L and 0 mg NO_2^--N /L. During Period III the fed COD/N ratio was 2.5 g/g but with a significant decrease of biomass concentration from Period II due to the removal of 5.25 g VSS to inoculate another reactor. The percentage of nitrogen removal was only 16-18% and concentrations in the effluent were around 5 mg NO_3^--N/L and 10 mg NO_2^--N /L, respectively. During the Period IV concentrations of ammonia around 180 mg NH_4^+-N/L were present in the effluent and inhibition of the nitrification by free ammonia (FA) ocurred (Anthonisen *et al*., 1976). Yang *et al*. (2004) observed that in the presence of concentrations of more than 18 mg NH_3-N/L might cause an inhibition of the production of exopolymers and with a detriment of biomass aggregation and

aerobic granules formation. This effect was not observed in the present study because the free ammonia concentration was kept between 4 and 8 mg NH_3-N/L. To avoid the inhibitory effect on the nitrifying activity due to free ammonia at high pH, the inlet ammonia concentration was decreased to 33 mg NH_4^+-N/L, causing a drop of the effluent concentration to 9 mg NH_4^+-N/L (Period V). In this period the nitrogen removal was around 40%. The NLR was increased again to re-establish the nitrifying activity, however, no increase of the nitrifying rate was observed (Period VI). The concentration of the inlet COD was decreased during period VII in order to favour the growth of the nitrifying bacteria. The nitrifying activity was restored before 50 days of operation in these conditions. Finally, an autotrophic medium was fed (Period VII), obtaining a fully stable nitrification to nitrate. Nevertheless, when the NLR was doubled nitrite appeared as the main product (Period VIII). During these three last periods no nitrogen removal was observed meaning that almost 100% of the fed ammonia nitrogen left the reactor in the form of ammonia, nitrite or nitrate.

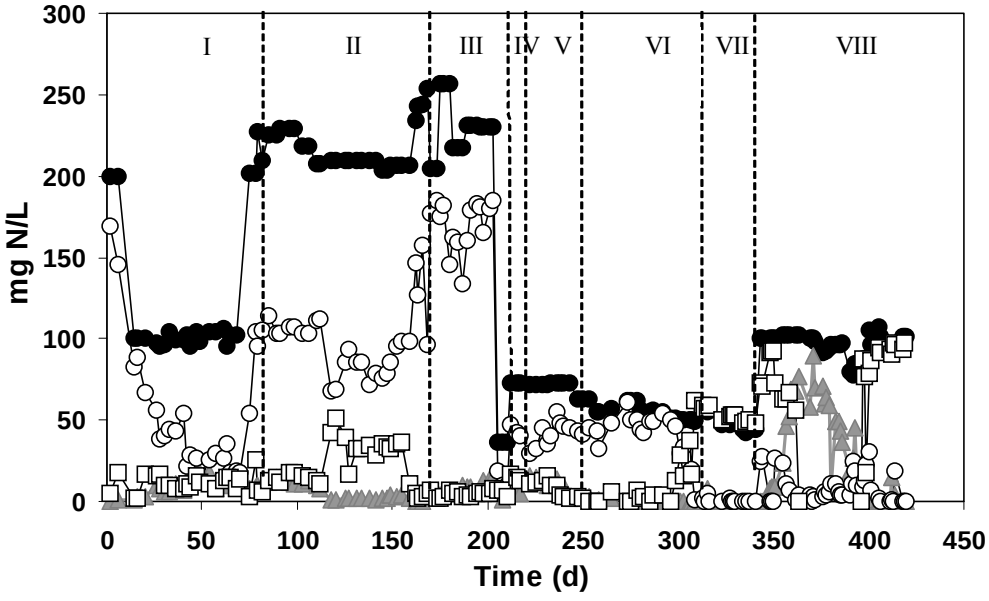


Figure 4.3. Ammonia concentration in the influent (●) and ammonia (○), nitrate (□) and nitrite (▲) concentrations in the effluent.

The organic loading rates (OLR) and the nitrogen loading rates (NLR) applied to the SBR during the experiment were in the range of 0 to 2 g COD/L·d and 0.14 to 0.80 g NH_4^+-N/L·d, respectively (Figure 4.4). The percentage of COD removal was almost constant (around 90%) while the nitrogen removal percentages were variable due to the changes of the operational conditions (Figure 4.5). The main processes for nitrogen removal in the SBR were nitrogen assimilation for biomass growth and nitrification-denitrification of ammonia. In order to discern between the percentages of nitrogen removal achieved by each of these mechanisms a nitrogen balance was calculated in the reactor to determine the amount of nitrogen used for growth.

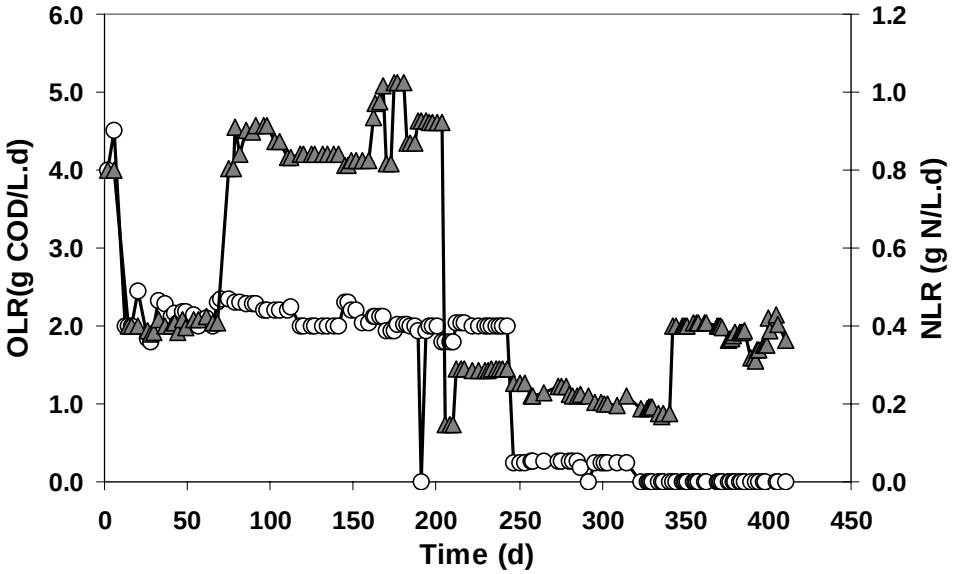


Figure 4.4. Nitrogen loading rate (▲) and organic loading rate (○) fed to the reactor.

The averaged percentages of nitrogen removed (N_R), calculated as the difference of the nitrogen amount in the feeding minus nitrogen in the effluent, and the expected percentages of assimilated nitrogen (N_A) are represented in Table 4.3. The fraction of nitrogen, removed by each mechanism, depended on the COD/N ratio of the influent in such a way that biomass assimilation can account for the removal of a large fraction of nitrogen when COD/N ratio in the influent to the SBR is high (Garrido *et al*., 2001). The fact that the granules exhibited different denitrifying activities depends on the COD/N ratio. As

occurred in biofilms O_2 is present only in the outer layers (Tijhuis *et al*., 1994; van Loosdrecht *et al*., 1995) and the inner layers, which were maintained under anoxic conditions, received the carbon source and nitrate to support the denitrification process. If the carbon source is not available enough (COD/N ratios low) the denitrification process is not completed and nitrite is produced as intermediate.

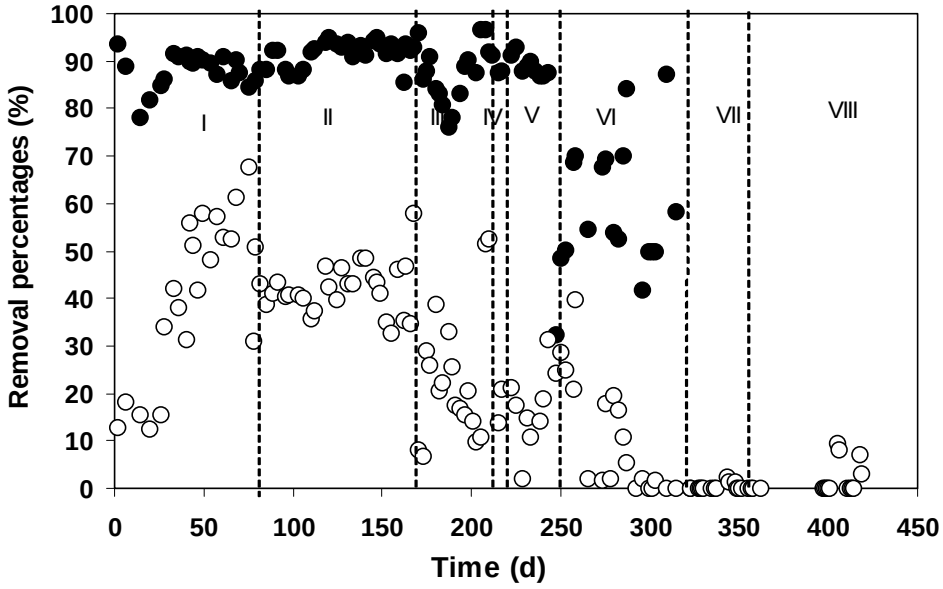


Figure 4.5. COD (●) and N(O) removal efficiency.

Table 4.3. Percentages of NH_4^+-N and NO_x^--N in the effluent and percentages of nitrogen removed (N_R), assimilated (N_A) and denitrified (N_D) referred to the inlet ammonia concentration.

Period	NH_4^+-N (%)	NO_x^--N (%)	N_R (%)	N_A (%)	N_D (%)
I	17-31	21-29	48-54	8	40-46
II	45-49	10-22	33-41	4	29-37
III	74-80	4-8	16-18	7	9-11
IV	24-27	20-22	53-55	39	14-16
V	48-68	22-34	10-18	15	0
VI	0	100	0	≈0	0
VII	0	100	0	≈0	0
VIII	3-9	91-97	0	≈0	0

The following figures from 4.6 to 4.9 show different profiles during a cycle of the SBR reactor. The figures show the evolution of the nitrogen compounds, COD and oxygen concentration along the operational cycle for each operational period.

In all of them the evolution of dissolved oxygen concentration (DO) and the COD reduction was similar. DO concentration was near the saturation value of 8 mg O_2/L. Almost all COD disappeared completely in all cases during the first 15 minutes of the cycle. The COD concentrations measured at the end of the cycle were similar in all cycles and around 25-40 mg/L.

However, related to the nitrogen compounds the performance was very different depending which was the COD/N tested. In general, nitrate was consumed via denitrification while COD might be partly aerobically oxidized, partly used as electron donor for denitrification and partly stored in the biomass. Ammonia was oxidised to nitrate during the aerobic period immediately after the disappearance of COD from the liquid phase.

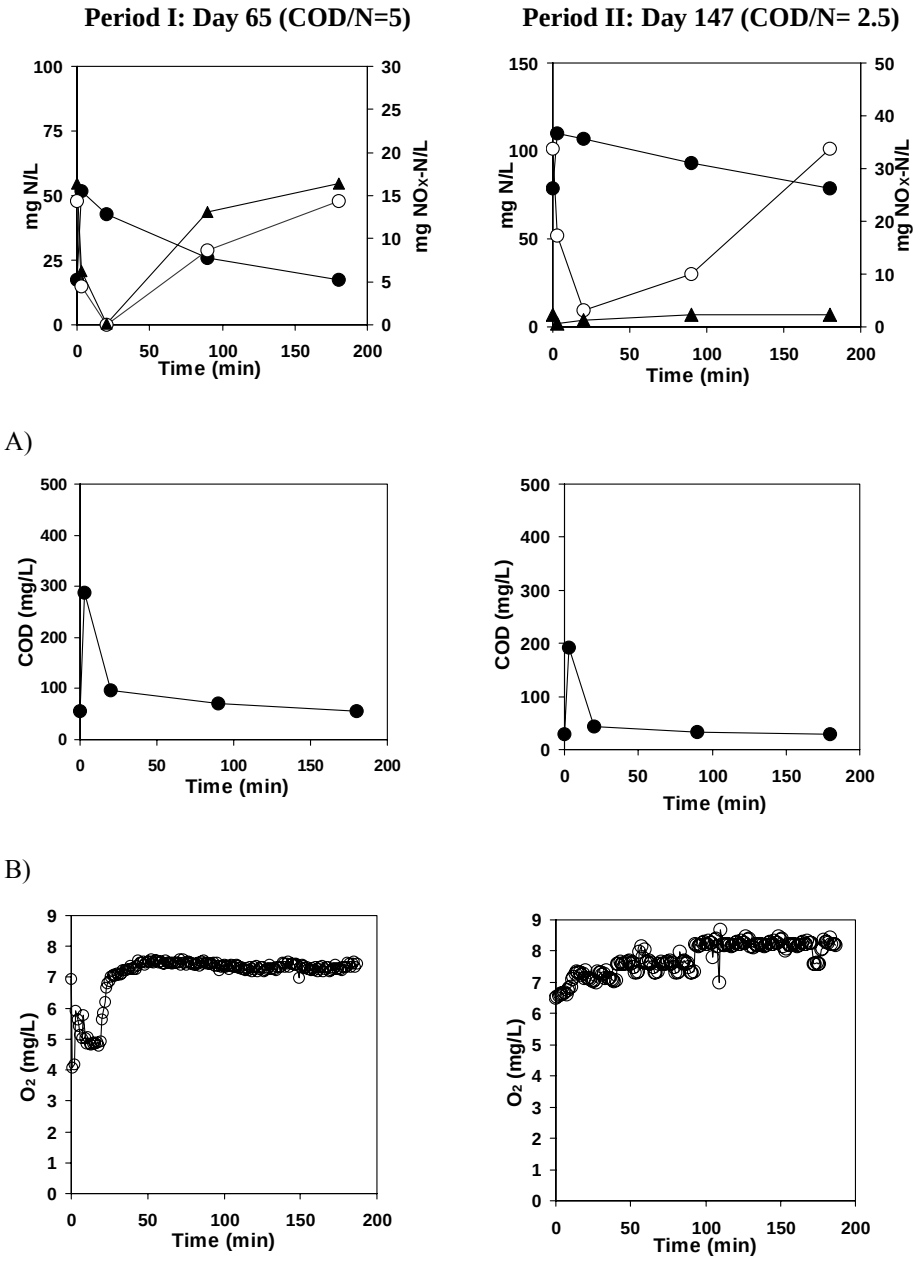


Figure 4.6. Typical concentration profiles during a cycle on day 65 and on day 147. A) (●)NH_4^+-N; (O) NO_3^--N; (▲)NO_2^- -N; B) (●)COD; C) Dissolved oxygen concentration.

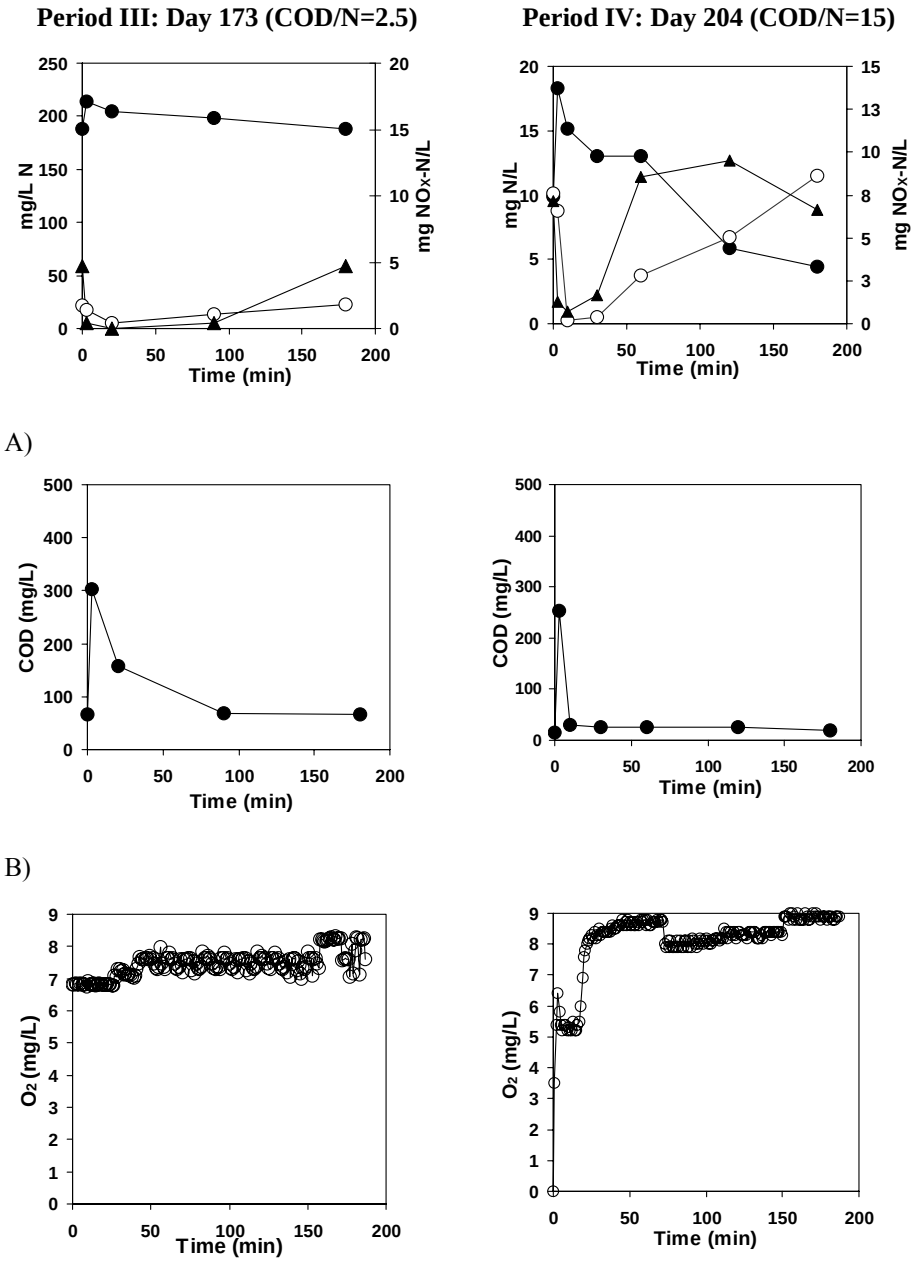


Figure 4.7. Typical concentration profiles during a cycle on day 173 and on day 204. A) (●)NH_4^+-N; (O) NO_3^--N; (▲)NO_2^- -N; B) (●)COD; C) Dissolved oxygen concentration.

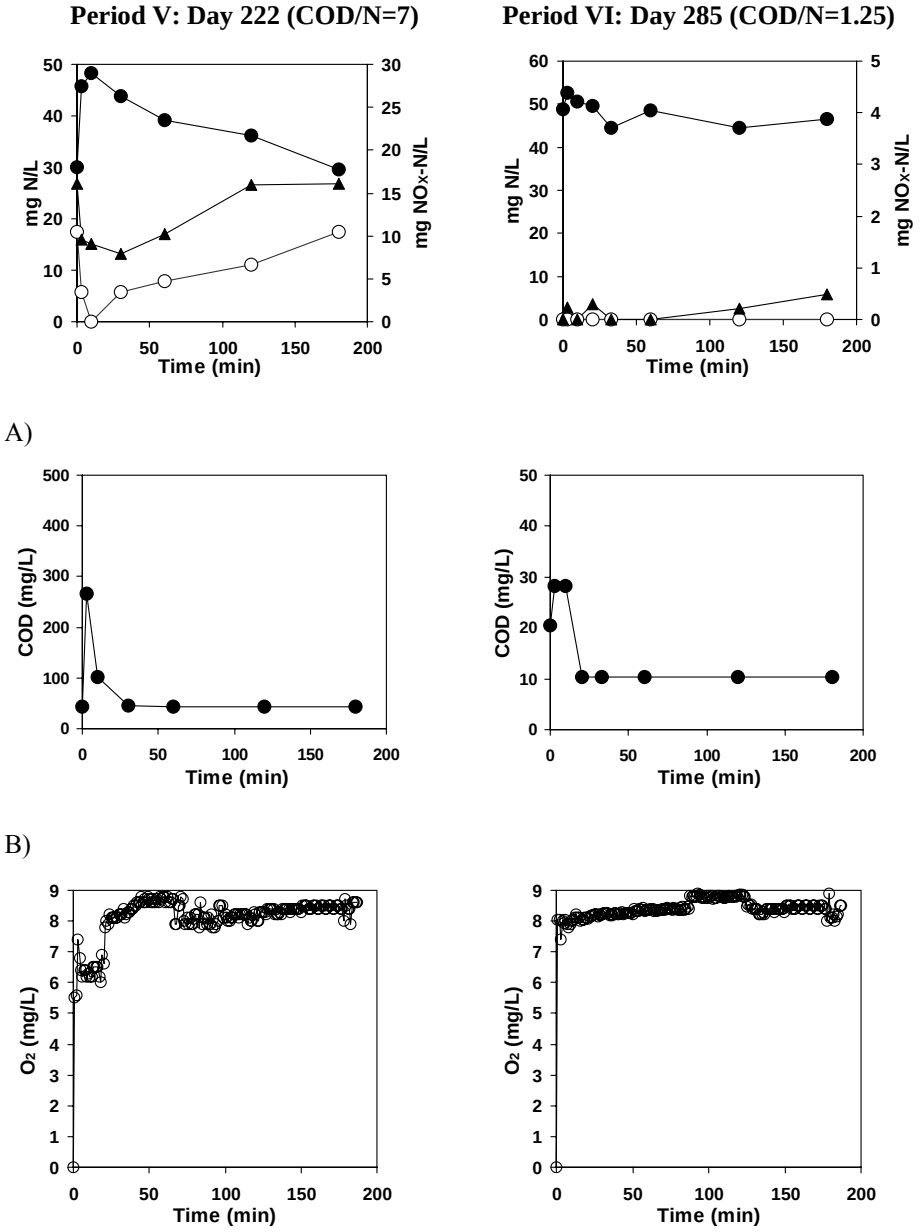


Figure 4.8. Typical concentration profiles during a cycle on day 222 and on day 285. A) (●)NH_4^+-N; (O) NO_3^--N; (▲)NO_2^- -N; B) (●)COD; C) Dissolved oxygen concentration.

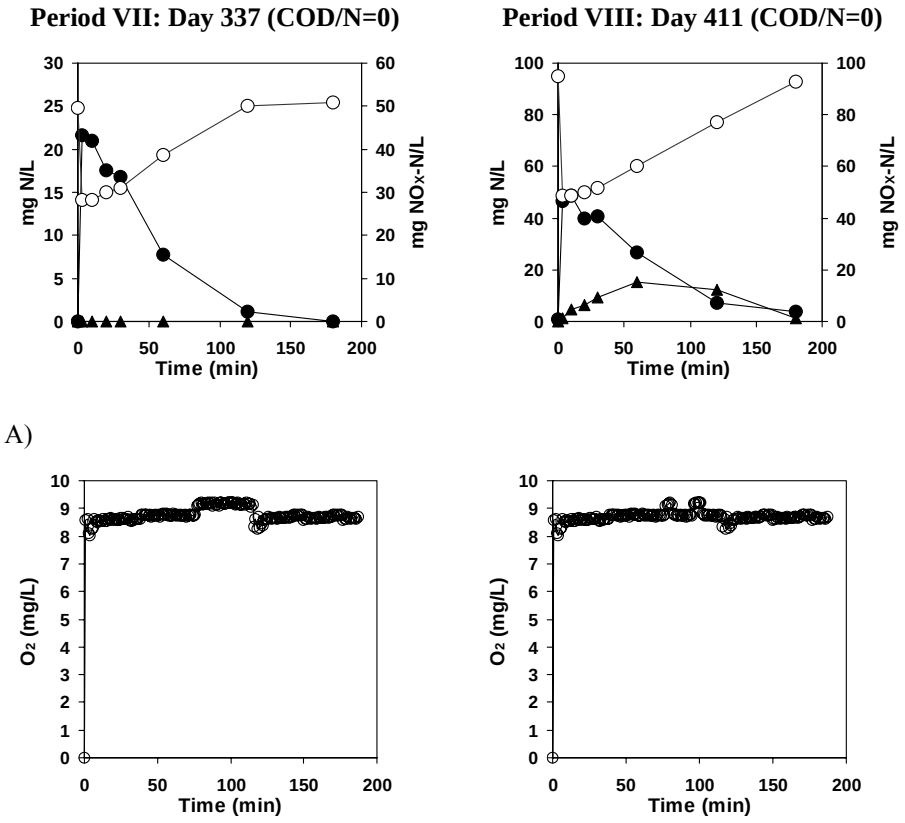


Figure 4.9. Typical concentration profiles during a cycle on day 337 and on day 411. A) (●)NH_4^+-N; (O) NO_3^--N; (▲)NO_2^- -N; B) Dissolved oxygen concentration.

Figure 4.10 show the nitrifying and denitrifying activities for each COD/N tested. Denitrifying and nitrifying specific activities were calculated from the cycle measurements as follows: the denitrifying activity from the nitrate consumption until depletion of this compound and the nitrifying activity from the slope of ammonia consumption after restoration of aeration The maximum denitrification specific activity obtained was of 1.1 g N/(g VSS·d) for COD/N ratio of 5, which is lower than those obtained in a previous work (Chapter 3), and the maximum nitrifying specific activity was around 0.033 g N/(gVSS·d), which was similar to those obtained in the previous work.

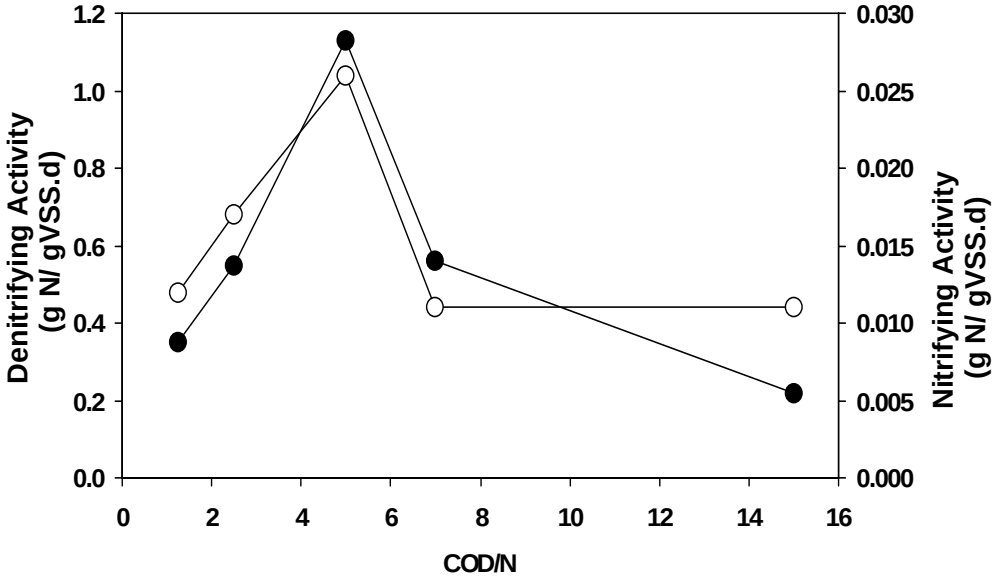


Figure 4.10. Influence of COD/N on denitrifying.(●; left axis) and nitrifying activities (O; right axis).

4.4.2. Biomass evolution

The concentration of the biomass increased from 3.5 to 9.0 g VSS/L (Figure 4.11) during Periods I and II when a constant inlet OLR of 2 kg COD /m^3·d was maintained. The biomass decrease observed in Period III is due to the removal of 5.25 g VSS. When the applied OLR was reduced to 0.25 kg COD /m^3·d (Period VI) and finally to zero (Periods VII and VIII) the biomass concentration

decreased from 7.5 to 1.0 g VSS/L due to the heterotrophs washout from the system. The biomass concentrations during the addition of COD in the influent were in the range of those obtained by Beun *et al*. (2002) operating a granular SBR at an OLR of 2.5 kg COD /m^3·d. On the other hand, the solids concentration in the effluent was always lower than 0.1 g VSS/L, this value decreased to 0.01 g VSS/L when no organic matter was applied to the reactor. This last value agrees with those obtained by Campos *et al.* (2000) who found solids concentrations in the effluent of 0.005-0.01 g VSS/L operating a nitrifying airlift reactor.

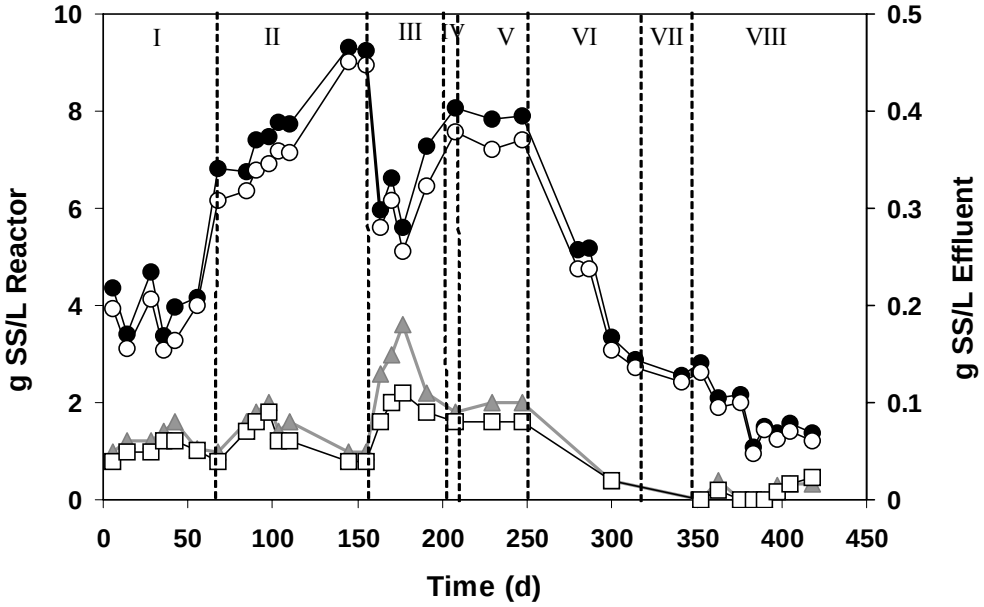


Figure 4.11. Concentration of VSS (○) and NVSS (●) in the system and concentration of VSS (□) and NVSS (▲) in the effluent.

The sludge volumetric index (SVI) trended to decrease during the first periods, achieving a stable value around 20 mL/g VSS (Figure 4.12). However during the autotrophic period there was a slight trend to increase. These low values indicate that the granules have a compact structure, which is related with a high settling velocity and a low requirement of volume after sedimentation (higher volumetric exchange ratio in the reactor). This agrees with Qin *et al*. (2004) who found that a decrease of the settling time caused a decrease of the

SVI. On the other hand, Tay *et al*. (2001) also observed the influence of the air velocity in the SVI value. These authors found that high hydrodynamic shear forces seemed to stimulate the production of cellular polysaccharides, which play an important role in the formation of aerobic granules. Although not enough information is given in this case about the O_2 concentration corresponding to each stage, which could be a limiting substrate when the hydrodynamic shear forces where lower due to less aeration and this produced granular sludge with bad settling properties due to this low O_2 concentrations.

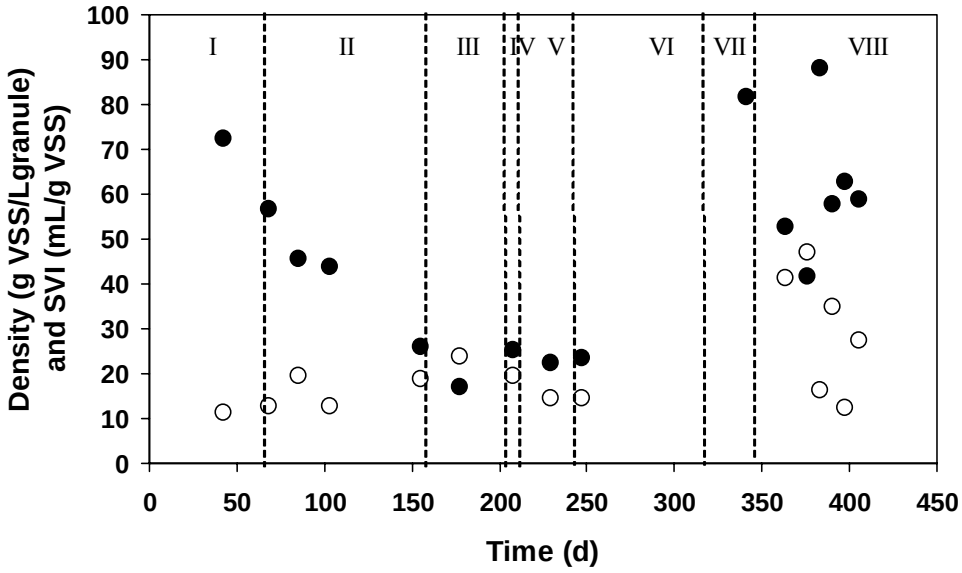


Figure 4.12. SVI (mL/g VSS) (●) and density (g VSS/L $_{granule}$) (○) of the biomass.

The density of the granules was between 10 and 30 g VSS/$L_{granule}$ and slightly increased to around 40 g VSS/$L_{granule}$ in period VIII when no organic carbon source was added and mainly nitrifiers were in the system. But due to the disappearance of the heterotrophs and their possible lysis the granules broke apart and the density decreased again with the consequent increase of SVI (Figure 4.13).

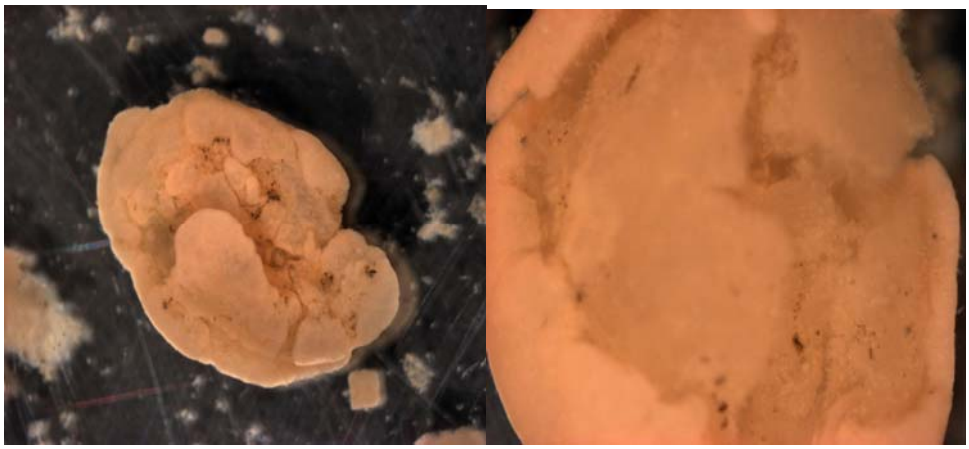


Figure 4.13. Autrotrophic granules broke in pieces during period VIII.

Obtained nitrifying granules maintained their settling properties in spite of the change in the composition performed in Periods VII and VIII due to complete removal of organic carbon source from the influent. The structure of granules changed and they appeared as empty granules looking like the shells of the original granules. To obtain nitrifying granules from sludge from a municipal WWTP long periods of time can be required due to the slow growth of the autotrophic biomass and the possible inhibition of the process by FA. Nitrifying granules can be easily obtained using the fast growing heterotrophic bacteria to form the initial structure of the granules where the nitrifying microorganisms grow retained in between the heterotrophs.

The growth of biofilms and granules is the result of mass transfer of substrate and subsequent conversion of the substrate by the biofilm. The outgrowth of a biofilm will be a function of the transport rate of the limiting substrate, the biomass yield and/or the biomass density in a biofilm. This implicates that a faster outgrowth of biofilms will occur at higher substrate surface loadings and for biofilms consisting of microorganism with a higher vield and forming a less dense biofilm. Research on nitrifying and heterotrophic bacteria show that under similar reactor conditions (temperature, shear) the nitrifiers form a much denser biofilms than heterotrophs (Tijhuis *et al*., 1994, 1995). Similarly slow growing methanogens are reported to form denser biofilms than fast growing acidifying bacteria. Althoug the density of biofilms is often

assumed to be related with the type of organisms present, there is evidence that hydrodynamic conditons also have an influence. Vieira *et al.* (1993) have shown that the density of a pure culture biofilm increases with increasing shear stress on the biofilm. Similar observations have been made in fluidized bed reactors (Chang *et al*., 1991) and in BAS reactors with nitrifying biofilms.

4.4.3. Biomass examination

Stereomicroscope images of the granules

The evolution of the granular biomass was followed by stereomicroscope observations. Pictures of the biomass along the operational period are shown in the Figure 4.14. The first one was taken during the period II when the granules had high percentages of heterotrophic bacteria. It can be observed that the granules were light brown and compacts.

During period VIII, it was observed that granules were hollow and could be described as "shell". From the initial granule, well compact, heterotrophs were presumably removed likely producing the biofilm breakage by the absence of organic matter and it were restocked by the nitrifying bacteria.

After COD removal from the feeding the granules were progressively changing from those compact and with smooth surfaces to others with open structures, dense in the outer layers but empty in the inside. Also small particles were observed in this period probably coming from the breakage of the big original granules.

From the stereomicroscopic observations is important to enhance the total absence of filament biomass and flocculent particles. The reactor only contained granular biomass.

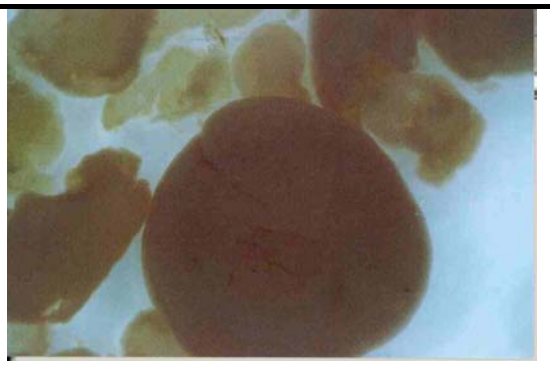
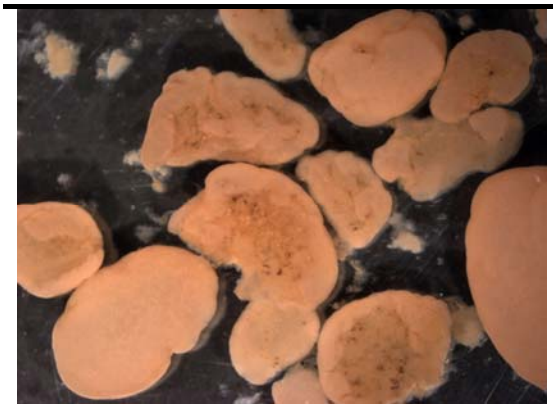
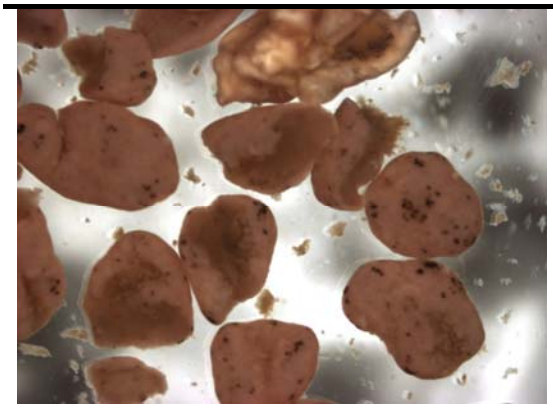
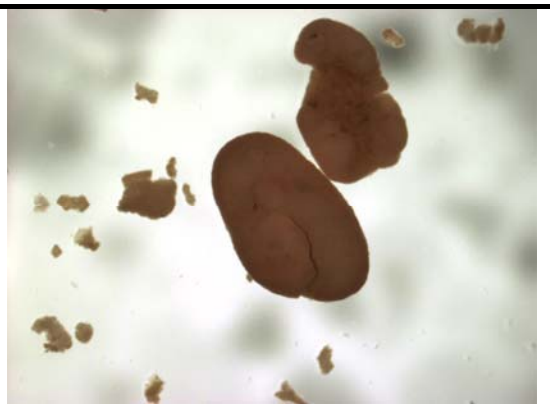
Heterotrophic granules 0,65X	***Operating day :150 1X***
	
Operating day:350 0,65X	***Operating day: 369 0,65X***
	
Operating day :417 0,65X	***Operating day: 426 0,65X***
	

Figure 4.14. Stereomicroscope images of the granules.

Scanning Electron Microscopy images of the granules

In order to observe the superficial structure of the granules scaning electron microscope images were taken.

Figure 4.15 represents some Scanning Electron Microscopy images of the granules on different operating days. It was shown complete granules (A), some details of the surface of granules (C, D, E and F) and the high biodiversity showing some organisms different of bacteria (B). The structure of the granules is shown in (A), where it can be observed the typical granular bacteria (Beun *et al.*, 1999). The figure represents a sample of mature granules with a cauliflower like aspect and shows a granule structure clearly distinguished. The sizes of the bacteria present in the granules are comprehended between 0.5 and 1.0 μm of diameter, which is a common value for this microorganism.

In the pictures of granules taken on operating days 340 and 430 it can be observed the aggregates of bacteria forming colonies and appear covered of a gelatine matter which could be as a matrix for her adhesion. This gelatine matter could be formed by extracellular polysaccharides (EPS) which are the responsible of the stable structure of aerobic granules.

In the literature (Wang *et al.*, 2005) showed that the outer shell of aerobic granule was composed of poorly soluble and non easily biodegradable EPS, whereas its core part was filled with readily soluble and biodegradable EPS. It was further found that the shell of aerobic granule exhibited a higher hydrophobicity than the core of granule. The insoluble EPS present in the granule shell would play a protective role with respect to the structure stability and integrity of aerobic granules.

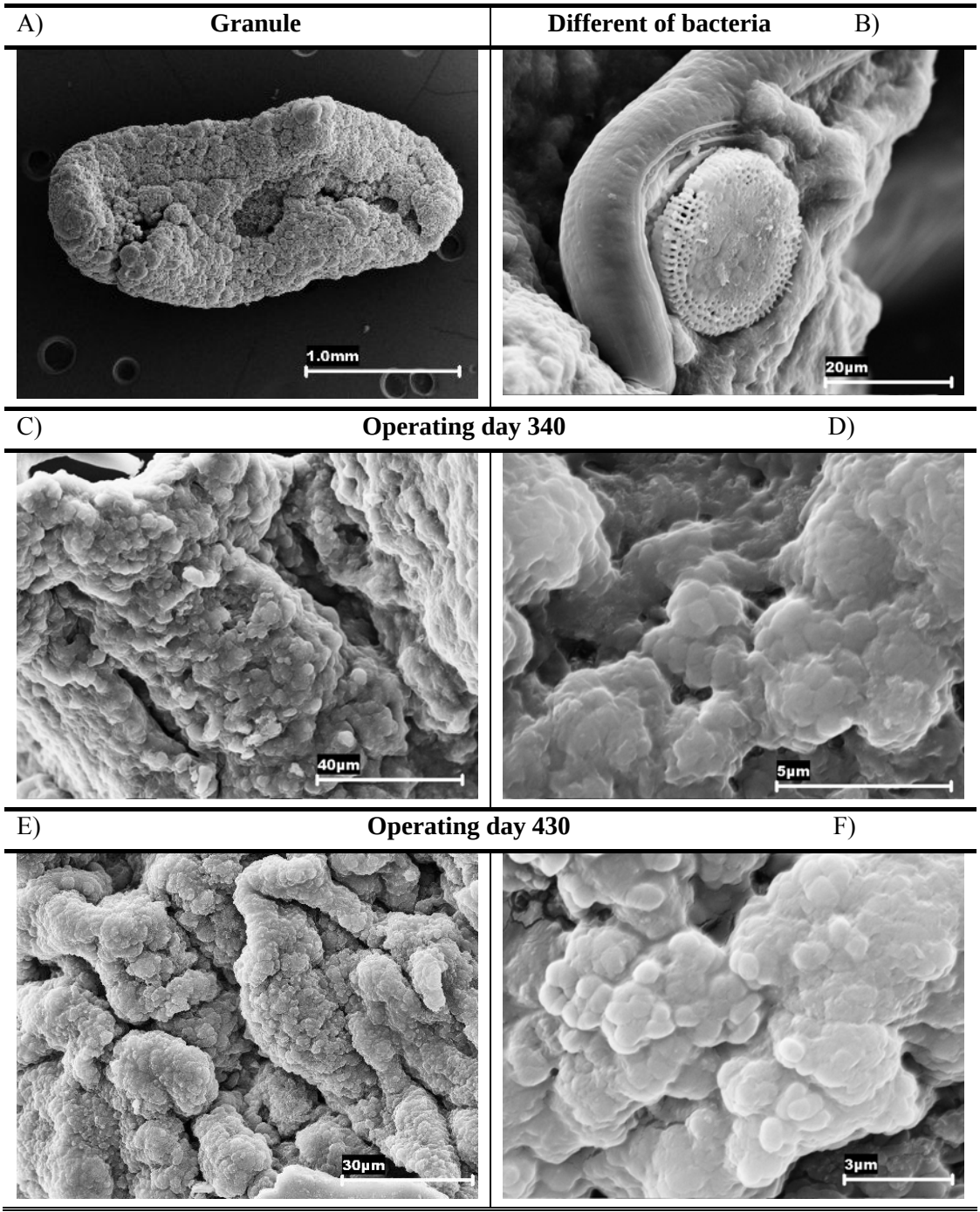


Figure 4.15. Scanning Electron Microscopy images of the granules on different operating days.

FISH (Fluorescence In Situ Hybridization)

Microbiological analyses were done during period VIII, by using samples of granules that were mechanically disrupted and analysed by the FISH technique.

Probes used for FISH were EUB338Mix, NEU653, which hybridize with most of ammonia-oxidizing bacteria (*Nitrosomonas* genus) and other two probes, which hybridize with most of nitrite-oxidizing bacteria. The NIT1035, which hybridize with the *Nitrobacter* genus bacteria and the Ntspa712, which hybridize with the *Nitrospira* genus bacteria (Table 4.4).

DAPI was applied in the Figure 4.16A and 4.16B, which dye of blue the entire DNA, while the *Nitrosomas* genus bacteria were shown in green. So, it can be observed that the majority of the DNA presents in the sample correspond to the ammonia-oxidizing bacteria.

In the Figure 4.16C can be observed that all of bacteria (probe EUB338Mix) were dyed of green, while in red gave a signal only the *Nitrospira* genus bacteria (probe Ntspa712). In the Figure 4.16D it were found dyed of red the *Nitrobacter* genus bacteria (probe NIT1035). It was observed that the nitrite-oxidizing population was lower than the ammonia-oxidizing bacteria.

Table 4.4. Oligonucleotide probes.

Probe	Probe sequence (5'→3')	% FA	Target organisms	References
EUB338I	GCT GCC TCC CGT AGG AGT	20	Bacteria domain	(Amann *et al.*, 1990)
EUB338II	GCA GCC ACC CGT AGG TGT	60	Bacterial lineages not covered by probe EUB338. Planctomycetales	(Daims *et al.*, 1999)
EUB338III	GCT GCC ACC CGT AGG TGT	60	Bacterial lineages not covered by probe EUB338 and EUB338II. Verrucomicrobiales	(Daims *et al.*, 1999)
NEU653	CCC CTC TGC TGC ACT CTA TTC CAT CCC CCT CTG CCG	40	Most halophilic and halotolerant Nitrosomonas spp.	(Wagner *et al*., 1995)
NIT1035	CCT GTG CTC CAT GCT CCG	40	Nitrobacter spp.	(Wagner *et al*., 1996)
	CCT GTG CTC CAG GCT CCG			
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	35	Most members of phylum *Nitrospira*	(Daims *et al*., 2001)
	CGC CTT CGC CAC CGG GTT CC			

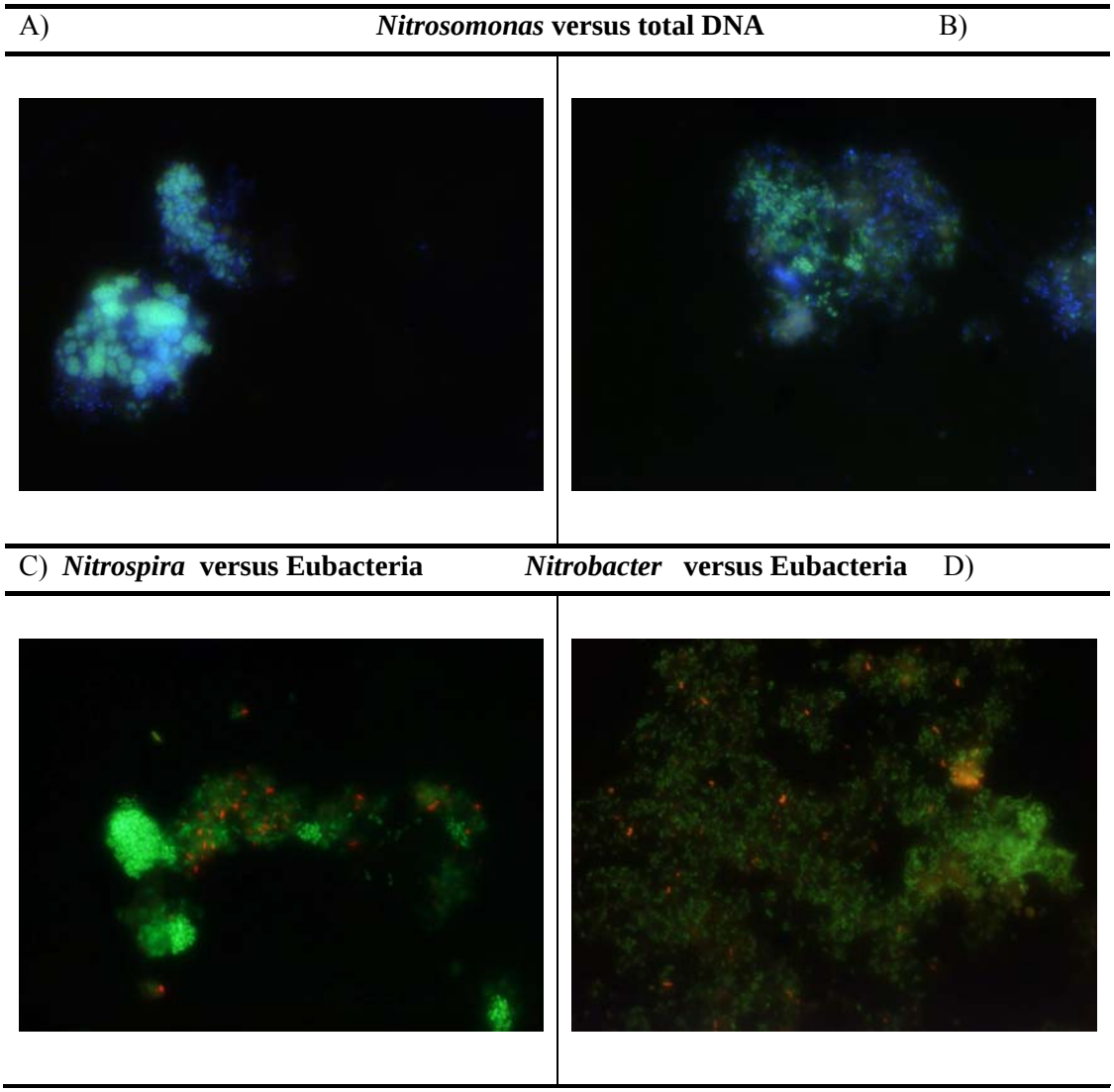


Figure 4.16. Pictures of granules biomass during period VIII. A) and B) in green *Nitrosomonas* genus and in blue DAPI dye. C) in green all bacteria and in red *Nitrospira* y D) in green all bacteria and in red *Nitrobacter*.

4.5. Conclusions

- An aerobic granular SBR was operated at different COD/N ratios achieving a removal of 90% for the organic matter and up to 55% for the ammonia nitrogen.
- The COD/N ratio influences the composition of the generated effluent by changing the extension of the different processes occurring in the granule. The higher the COD/N ratio up to 5 the higher the N removal percentages.
- Nitrifying granules are easily obtained by removing the organic carbon source from the feeding to an aerobic granular SBR where heterotrophic organic matter oxidation, denitrification and nitrification processes take place.

4.6. References

Akunna J.C., Bizeau C. and Molletta R. (1993). Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose glycerol acetic acid lactic acid and methanol. *Water Research*, **27**(8), 1303-1312.

Amann R. I., Binder B. J. , Olson R. J., Chisholm S. W., Devereux R., and Stahl D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flowcytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, **56**, 1919–1925.

APHA-AWWA-WPCF. (1999). Standard Methods for the examination of water and wastewater. 20th Edition. Clesceri, L.S., Greenberg, A.E. & Eaton, A.D. (eds.).

Anthonisen A.C., Loehr R.C., Prakasam T.B.S. and Srinath E.G. (1976). Inhibition of nitrification by ammonia and nitrous acid. *Journal WPCF*, **48**(5), S35-S52.

Beun J.J., Hendriks A., van Loosdrecht M.C.M., Morgenroth E., Wilderer P.A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research,* **33** (10), 2283-2290.

Beun J.J., Heijnen J.J. and van Loosdrecht (2001). N-removal in a granular sludge sequencing batch airlif reactor. *Biotechnology and Bioengineering,* **75**, 1, 82-92.

Beun J.J., van Loosdrecht M.C.M. and Heijnen J.J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research*, **36**, 702-712.

Buys B.R., Mosquera-Corral A., Sánchez M. and Méndez R. (2000). Development and application of a denitrification test based on gas production. *Water Science and Technology*, **41** (12), 113-120.

Campos J.L., Méndez R. and Lema J.M. (2000). Operation of a nitrifying activated sludge airlift (NASA) without biomass carrier. *Water Science and Technology*, **41**(4-5), 113-120.

Carley B.N, and Mavinic D.S. (1991). The effects of external carbon loading on nitrification and denitrification of high ammonia landfill leachate. *Journal Water Pollution Control Federation*, **63**, 51-59.

Cassidy D.P. and Belia E. (2005). Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. *Water Research*, **39**, 4817-4823.

Chang H. T., Rittmann B.E., Amar D., Hein R., Ehrlinger O. and Lesty Y. (1991). Biofilm detachment mechanism in a liquid fluidized bed. *Biotechnology Bioengineering*, **38**, 499-506.

de Beer D., van den Heuvel J.C. and Ottengraf S.P.P. (1993). Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. *Applied and Environmental Microbiology*, **59**(2), 573-579.

Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology,* **22,** 434-444.

Daims H., Nielsen J. L., Nielsen P. H., Schleifer K. H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. *Applied and Environmental Microbiology*, **67**, 5273-5284.

Garrido J. M., Omil F., Arrojo B., Méndez R. and Lema J.M. (2001). Carbon and nitrogen removal from a wastewater of an industrial dairy laboratory with a coupled anaerobic filter-sequencing batch reactor system. *Water Science and Technology,* **43**, 315–321.

Jang A., Yoon T.H., Kim I.S., Kim K.S. and Bishop P.L. (2003). Characterization and evaluation of aerobic granules in sequencing batch reactor. *Journal of Biotechnology*, **105**, 71-82.

Jiménez B., Noyola A., Capdeville V., Roustan M. and Faup G. (1988) Dextran Blue coulorant as a reliable tracer in submerged filters. *Water Research,* **22**, 1253-1257.

Komorowska-Kaufman M., Majcherek H. and Klaczynski E. (2006). Factors affecting the biological nitrogen removal from wastewater. *Process Biochemistry*, **41**, 1015-1021.

Knowles R. (1982). Denitrification. *Microbiological Reviews,* **46** (1), 43-70.

Lin Y.M., Liu Y. and Tay J.H. (2003). Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. *Applied Microbiology and Biotechnology*, **62**, 430-435.

Mateju V., Cizinka S., Krejci J. and Janoch T. (1992). Biological water denitrification-a review. *Enzyme Microbial Technology*,**14**, 170-183.

Qin L., Tay J.H. and Liu Y. (2004). Selection pressure is a driving force of aerobic granulation in sequencing batch reactors. *Process Biochemistry*, **39**, 579-584.

Polprasert C. and Park H.S. (1986). Effluent denitrification with anaerobic filter. *Water Research*, **20** (8), 1015-1021.

Sánchez M., Mosquera-Corral A., Méndez R. and Lema J.M. (2000). Simple methods for the determination of the denitrifying activity of sludges. *Bioresource Technology*, **75**, 1-6.

Smolders G.J.F., Klop J., van Loosdrecht M.C.M. and Heijnen J.J. (1995). A metabolic model of the biological phosphorus removal process. Effect of the sludge retention time. *Biotechnology and Bioengineering,* **48**, 222-233.

Soto M., Veiga M.C., Méndez R. and Lema J.M. (1989). Semi-micro COD determination method for high salinity wastewater. *Environmental Technology Letters,* **10**(5), 541-548.

Tam N.F.Y., Wong Y.S. and Leung G. (1992). Effect of exogenous carbon sources on removal of inorganic nutrient by the nitrification-denitrification process. *Water Research*, **26**(9), 1229-1236.

Tay J-H., Lui Q-S. and Liu Y. (2001). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology*, **33**, 222-226.

Tay J-H., Liu Q-S. and Liu Y.(2002). Aerobic granulation in sequential sludge blanket reactor. *Water Science and Technology*, **46** (4), 13-18.

Tijhuis L., van Loosdrecht M.C.M. and Heijenen J.J. (1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioengineering*, **44**, 595-608.

Tijhuis L., Huisman J.L, Hekkelman H.D., van Loosdrecht M.C.M. and Heijnen J.J. (1995). Formation of nitryfing biofilms on small suspended particles in airlift reactors. *Biotechnology Bioengineering*, **47**(5), 585-595.

Tokutomi T. (2004). Operation of a nitrite-type airlift reactor at low DO concentration. *Water Science and Technology*, **49** (5-6), 81-88.

Tsuneda S., Nagano T., Hoshima T. Ejiri Y., Noda N., and Hirata A. (2003). Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Research*, **37**, 4965-4973.

Tsuneda S., Ogiwara M., Ejiri Y. and Hirata A. (2006). High-rate nitrification using aerobic granular sludge. *Water Science and Technology*, **53** (3), 147-154.

van Benthum W.A.J., van Loosdrecht M.C.M. and Heijnen J.J. (1997). Control of heterotrophic layer formation on nitrifying biofilms in a biofilm airlift suspension reactor. *Biotechnology and Bioengineering,* **53**, 397-405.

van Loosdrecht M.C.M., Tijhuis L., Widieks A.M.S. and Heijnen J.J (1995). Population distribution in aerobic biofilms on small suspended particles. *Water Science and Technology,* **31**, 163–171.

Vieira M.J., Melo L.F. and Pinheiro M.M. (1993). Biofilm formation: hydrodynamic effects on internal diffusion and structure. *Biofouling*, **7**, 67-80.

Wang Z.W., Liu Y. and Tay J.H. (2005). Distribution of EPS and cell surface hydrophobicity in aerobic granules. *Applied Microbiology Biotechnology*, **69**, 469-473.

Wagner M., Rath G., Amann R., Koops H.-P. and Schleifer K.-H. (1995). In situ identification of ammonia-oxidizing bacteria. *Systematic in Applied Microbiology*, **18**, 251-264.

Wagner M., Rath G., Koops H.P., Flood J. and Amann R. (1996). In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*, **34**, 237-244.

Yang S.F., Tay J.H. and Liu Y. (2003). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *Journal of Biotechnology*, **106**, 77-86.

Yang S.F., Tay J.H. and Liu Y. (2004). Inhibition of free ammonia to the formation of aerobic granules. *Biochemical Engineering Journal*, **17**, 41-48.

Chapter 5

Effects of the hydrodynamic conditions and feeding composition on the aerobic granulation in sequencing batch reactors[1]

Summary

The effect of shear force on aerobic granulation was studied in a sequencing batch reactor with an H/D ratio of 2.5. Hydrodynamic turbulence caused by upflow aeration and mechanical stirrer as the main shear forces in the system. Compact and regular aerobic granules were formed in the reactor with a superficial upflow air velocity of 1.58 cm/s.

The second objective was to study the effect of different carbon to nitrogen ratios (TOC/N) in the feeding on the production of nitrogen compounds in the effluent and the TOC concentration in the influent on the granulation process. The feeding flow was a synthetic medium, which contained acetate as carbon source (37.5-190 mg TOC/L) and ammonium as N source (25-50 mg NH_4^+-N/L). Different TOC/N ratios of 7.5, 3.0, 1.50 and 0.75 g/g in the feeding were tested. The TOC removal percentage was around 80-95% during the whole operational period. The N removal percentages obtained in the reactor were up to 40%. Removal of ammonia was carried out by assimilation process.

Part of it submitted to:

[1]**Arrojo B., Figueroa M., Mosquera-Corral A., Campos J.L. and Méndez R.** (2006). Effects of hydrodynamic conditions and feeding composition on the aerobic granulation in SBRs.

5.1. Introduction

Aerobic granulation can be regarded as the gathering together of cells through cell-to-cell immobilization to form a stable, contiguous, multicellular association. These aggregated granules have a compact structure as compared wiith suspended sludge flocs (Tay *et al*., 2002a; 2002b). Studies showed that aerobic granulation is a gradual process from seed sludge to compact aggregates, further to granular sludge and finally to mature granules (Liu and Liu, 2006). Obviously, for cells in a culture to aggregate, a number of factors could affect this process. For example, the hydrodynamic shear force, the reactor configuration and the feeding composition (Tay *et al*., 2006; de Kreuk and van Loosdrecht, 2006; Tay *et al*., 2003).

Hydrodynamic Shear Force

In a bubble column SBR, hydrodynamic shear force is mainly created by aeration that can be described by the upflow air velocity. A study showed that higher shear force favoured the formation of more compact and denser aerobic granules, while the stimulated production of extracellular polysaccharides and the microbial activity at high shear force was also observed (Tay *et al*., 2001b; Liu and Tay, 2002). It is well know that extracellular polysaccharides can mediate both cohesion and adhesion of cells and play a crucial role in maintaining structural integrity in a community of immobilized cells (Liu *et al*., 2004). Consequently, the enhanced production of extracellular polysaccharides at high shear force can make granule structure more compact and stronger.

In biofilm system, high detachment force favourable to the formation of compact and thin biofilm has been widely reported. It was thought that the balance between biomass growth and detachment by shear force is key to the stability of biofilm (Trinet *et al*., 1991; Chang *et al*., 1991; van Loosdrecht *et al.*, 1995; Kwok *et al*., 1998). In addition, it was found that detachment was strongly associated with biomass growth rate.

Similar to the formation of a biofilm, aerobic granules can form at different levels of hydrodynamic shear forces. Therefore, hydrodynamic shear force is not a primary inducer of aerobic granulation in SBR (Liu and Tay, 2002). However,

the structure of mature aerobic granules is hydrodynamic shear force-related (Liu and Tay, 2006). High shear in terms of superficial upflow air velocity could lead to more compact, denser, rounder, stronger and smaller aerobic granules (Chang *et al.*, 1991; Shin *et al.*, 1992; Tijhuis *et al.*, 1994; van Benthum *et al*., 1997; Gjaltema *et al.*, 1997; Tay *et al.*, 2001a; Liu and Tay, 2002; Wang *et al.*, 2005; Tay *et al*., 2004).

Reactor Configuration

Aerobic granules have been produced only in column SBR so far. It can be understood that reactor configuration has an impact on the flow patterns of liquid and microbial aggregates in the reactor. In a column SBR, air flow is subject o an upflow pattern. The air or liquid upflow pattern in a column reactor creates a relatively homogenous circular flow and localized vortex along the reactor height; and thus microbial aggregates are constantly subjected to circular hydraulic attrition (Liu and Tay, 2002).

The feasibility and efficiency of other types of bioreactors, such as completely mixed tank reactor (CMTR) in development of aerobic granular sludge have not been sufficiently demonstrated so far. In a hydrodynamic sense, column-type upflow reactor and CMTR have very different hydrodynamic behaviours in terms of interactive patterns between flow and microbial aggregates. According to the thermodynamics, the circular flow could force microbial aggregates to be shaped as regular granules that have a minimum surface free energy, provided those aggregates could be kept in the reactots under given dynamic conditions. Thermodynamically, such a phenomenon is very similar to the formation of benthic round-shape boulders in a natural flowing river system. It is obvious that in a column type upflow reactor a higher ratio of reactor height to diameter can ensure a longer circular flowing trajectory, which in turn creactes a more effective hydraulic attrition to microbial aggregates. However, in CMTR microbial aggregates stochastically move with dispersed flow in all directions. Thus, microbial aggregates are subject to varying localized hydrodynamic shear force, flowing trajectory and random collision. Under such circumstances, only flocs of irregular shape and size instead of regular granules occasionally form, and this is exactly like what happens in a conventional

activated sludge aeration tank, which is a typical CMTR.

It seems certain that not only the strength of hydrodynamic shear force, but also the interactive pattern between flow and microbial aggregates have effects on the formation of granular sludge. In this aspect, the column-type upflow reactor with high ratio of reactor height to diameter can provide an optimal interactive pattern between flow and microbial aggregates for granulation. It can ensure a circular flowing trajectory, which in turn creates a more effective hydraulic attrition for microbial aggregates. A high H/D ratio may also improve oxygen transfer and could result in a reactor with a small footprint (Beun *et al*., 2002; de Bruin *et al*., 2004)). This may be a major reason why almost all of the granular sludge only forms in column-type upflow reactors. In an engineering sense, the desirable interactive pattern between flow and aggregates might be achieved by controlling reactor configurations and operation strategy. Consequently, a better understanding of the role of flow pattern in granulation process would lead to the development of novel types of granular sludge reactor.

Feeding composition

Studying the possibility of forming granules on wastewater with low organic matter composition in a sequencing batch reactor was a logical step in the scaling-up process and development of this technology. de Kreuk *et al.* (2006) studied the formation of aerobic granules with domestic sewage and they found that the chemical oxygen demand (COD) load could be a critical factor for granulation. Domestic sewage typically has a much lower content than industrial wastewater and it can be expected that this will influence the granule formation process (Moy *et al*., 2002).

Tay *et al*. (2003) demonstrated that the organic loading rate (OLR) affected the formation of aerobic granules. They found that there were no granules formed under the OLR of 1 kg COD/m^3·d. Instead, flocs with rather loose structure dominated reactor mixed-liquor. They also found that the mixed liquor was always a mixture of granules and flocs when the OLR was 8 kg COD/m^3·d. As a result, too high or too low OLR appeared to be unfavourable for the formation of a compact sludge bed, and further, for maintaining the stability of the reactor.

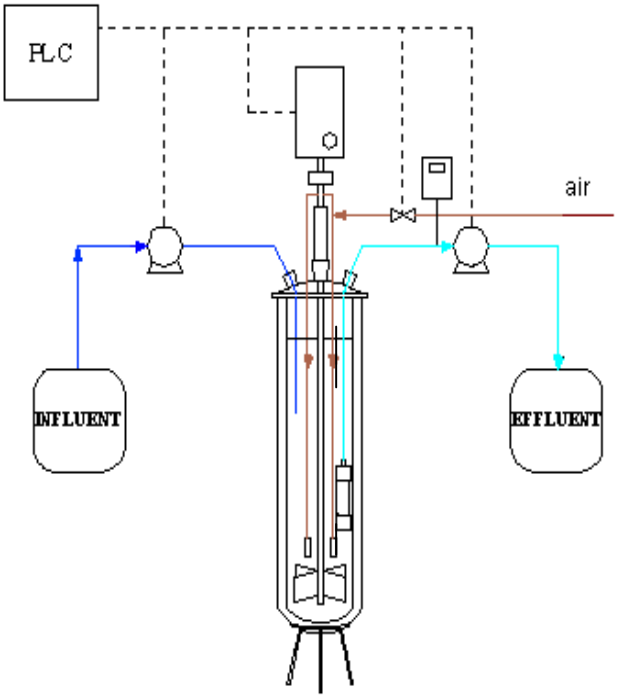
5.2. Objectives

- To study the effect of the reactor configuration (H/D ratio) and the hydrodynamic shear force in the formation and operation of the aerobic granular sludge.
- To study the effect of the composition of the feeding media in terms of its C/N ratio on the removal efficiencies of organic matter and nitrogen compounds in a granular SBR.
- To study the effect of TOC in the influent on the granulation process and performance.

5.3. Materials and methods

5.3.1. Experimental set-up

A sequencing batch reactor (SBR) with a total volume of 5 L and a working volume of 3 L was used. Dimensions of the units were: total height of 0.6 m and inner diameter of 0.12 m. The height to the diameter ratio (H/D) being 2.5 (Table 5.1). Oxygen was supplied to the reactor by using spargers to promote the formation of small air bubbles. The flow of air was controlled by means of electrovalves at 10.87 L/min. The used mechanical stirrer was a Rushton turbine, with a standard 6-blade disk impeller and was operated at rotating speed of 100 rpm. A set of two peristaltic pumps was used to feed the influent and to discharge the effluent. The influent was introduced in the system through port located at the top of the reactor. The effluent was discharged through the sampling port placed at middle height of the column reactor. A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations over the pumps and valves, and thus the length of every operational phase in the operational cycle of the SBR (Figure 5.1). The reactor was operated at room temperature (15-20 ºC) and without pH control, which varied between 7.4 and 8.5. The dissolved oxygen concentration was maintained around 5 mg O_2/L and 9 mg O_2/L during the feast and famine periods, respectively.



A)

B)

Figure 5.1. A) Experimental set up B) Picture of the reactor.

Table 5.1. Dimensions of the SBR.

Parameter	*Value*
Total volume (L)	5.0
Working volume (L)	3.0
Total height (m)	0.60
Used height (m)	0.30
Inner diameter (m)	0.12
Impeller diameter (m)	0.06
Impeller height (m)	0.09
H/D ratio	2.5

5.3.2. Inocula

The sludge used to inoculate the SBR was the typical flocculent activated sludge with a fluffy, irregular and loose morphology and relative abundance of filamentous microorganisms (Figure 5.3). Settling properties of this sludge were: SVI of 100 mL/g VSS, and ZSV of 0.25 m/h.

5.3.3. Feeding media

The reactor was fed with the synthetic media described in Table 5.2. The composition of the synthetic wastewater fed to reactor was according to Beun *et al*. (1999) and the trace solution to Smolders *et al*. (1995). The synthetic wastewater contained soluble COD as the sole organic matter and ammonium as N source.

Table 5.2. Composition of the synthetic media and traces solution.

Feeding	
Compound	**Conc. (g/L)**
$CH_3COONa{\cdot}3\ H_2O$	0.20-1.06
NH_4Cl	0.1-0.20
K_2HPO_4	0.092
KH_2PO_4	0.036
$MgSO_4$	0.049
KCl	0.019
Traces solution	0.5 mL/L

Traces solution	
Compuesto	**Conc. (g/L)**
$FeCl_3{\cdot}6\ H_2O$	1.5
H_3BO_3	0.15
$CoCl_2{\cdot}6\ H_2O$	0.15
$MnCl_2{\cdot}4\ H_2O$	0.12
$ZnSO_4{\cdot}7\ H_2O$	0.12
$NaMoO_4{\cdot}2\ H_2O$	0.06
$CuSO_4{\cdot}5\ H_2O$	0.03
KI	0.03

5.3.4. Strategy of operation

The operational conditions of the reactor are summarized in Table 5.3. Different TOC/N ratios of 7.5, 3.0, 1.5 and 0.75 g/g in the feeding were used. The system was operated in cycles of 3 hours with an exchange volume of 50% (Figure 5.2). The hydraulic retention time (HRT) was always 0.25 days.

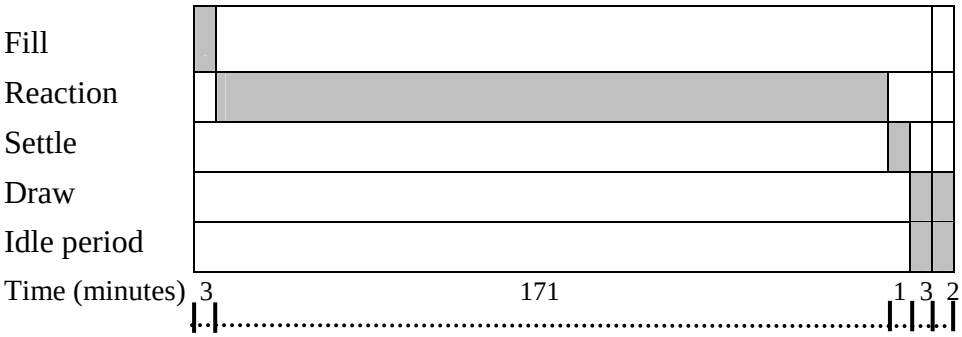


Figure 5.2. Operation strategy of the SBR during a cycle.

Table 5.3. Operational parameters during the different stages of the SBR.

Stage	Days (d)	TOC (mg/L)	NH_4^+-N (mg/L)	TOC/N (g/g)
I	0-120	190	25	7.5
II	120-150	75	25	3.0
III *	150-190	37.5	25	1.5
IV	190-220	37.5	50	0.75

* Inoculation of nitrifying sludge.

5.3.5. Analytical methods

The pH, dissolved oxygen, nitrate, ammonia, TOC, volatile suspended solids (VSS), total suspended solids (TSS), zone settling velocity (ZSV) and sludge volumentric index (SVI) were determined accordingly to Standard Methods (APHA, 1999) as described in Chapter 2. Concentration of chemical oxygen demand (COD) was determined by a modified method from the Standard Methods (Soto *et al*., 1989). The morphology and size distribution of the granules was measured regularly by using an Image Analysis procedure proposed by Tijhuis *et al*. counting a sample of more than 200 granules (Tijhuis *et al*., 1994). Biomass density, in terms of g VSS per litre of granules, was determined with dextran blue, which is not absorbed by the biomass (Jiménez *et al*. 1988) and following the methodology proposed by Beun (Beun *et al*., 1999) according to section 2.3 of Chapter 2. The presence of nitrifying bacteria in granules was followed by Fluorescence *in situ* hybridization (FISH) (Section 2.4 of Chapter2). This analysis was performed with a set of fluorescent labelled 16S rRNA-targeted DNA probes according to the procedure described by Amann (1995). The used probes for in situ hybridization were labelled with the dyes FITC or Cy3 and in all cases the reagent DAPI was also applied to detect the entire present DNA in the samples. Fluorescence signals of disaggregated samples were observed under an Axioskop 2 epifluorescence microscope (Zeiss, Germany) provided with a digital camera (Coolsnap, Roper Scientific Photometrics) and images were collected in a computer.

5.4. Results and discussion

In the next section the main results obtained from the operation of the reactor are shown and discussed. The physical characteristics of the granules and the removal efficiencies of nitrogen and carbon compounds together with the effluent quality with respect to the concentration of suspended solids were analyzed.

5.4.1. Biomass granulation process

During the first seven experimental days an almost complete washout of the suspended biomass in the system was observed (Figure 5.3). As it was observed in previous works this was a result of the operation strategy of the systems, in which a very short settling and a fast effluent withdrawal period were applied to the reactor. Thus, either flocs or aggregates of biomass with settling velocity slower than 9 m/h were removed from the system as a result of mentioned conditions. Two weeks after the start up of the reactors the formation of small aggregates with an average diameter of 0.5 mm was observed in the system. Suspended flocs gradually disappeared from the reactor and settling properties of the obtained aggregates were very good, SVI 50 mL/g VSS and ZSV of 10 m/h. Microscopic examination of the sludge showed that the morphology of the granular biomass was completely different from the flocculent sludge that was used as inoculum. The shape of the granules was round with a cauliflower like aspect and very clear outline (Figure 5.3)**.**

The granular size distribution along the operational time was monitored and a gradual increase with time of operation was observed (Figure 5.3; 5.4). These results indicated that the formation of aerobic granules was a progresive process from the flocculent seeded sludge to compact aggregates, further to granular sludge, of 0.75 mm after three weeks of operation, and finally to mature granules of 1.75 mm of averaged diameter (Figure 5.5). The evolution of size distributions of the granules is shown for days from 8 to 132 in terms of volume percentage. Most of the volume percentage of the biomass on day 7 corresponded to granules with a size distribution between 0.05 and 1.25 mm. However, from day 50 on, the volume percentage was shifted to diameters between 1.25 and 3.65 mm indicating the larger contribution of the big granules to the biomass concentration in the system.

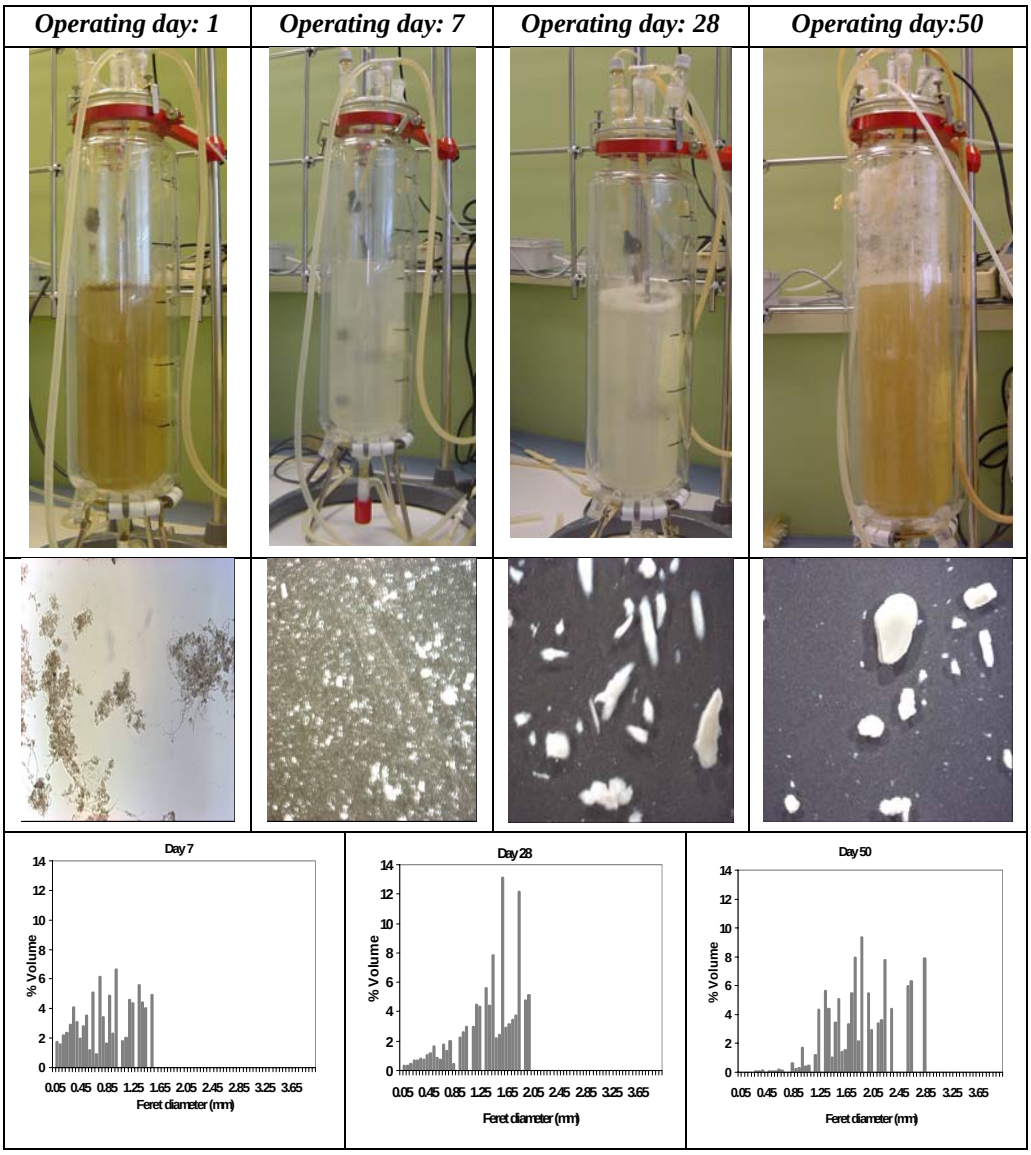


Figure 5.3. Pictures of the reactor, sizes distribution and stereomicroscopic images of the seeding sludge (100x) (Day 1) and the aerobic granules on different operating days, 1, 7, 28 and 50. (6.5x).

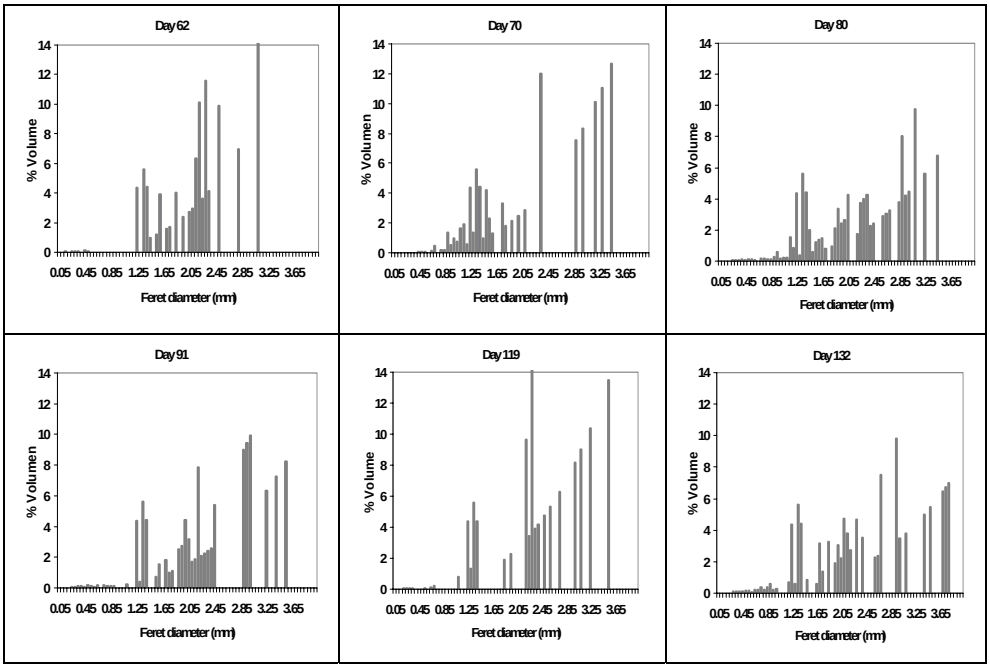


Figure 5.4. Comparison of the size distribution between granular sludge on operating days 62, 70, 80, 91, 119, 132.

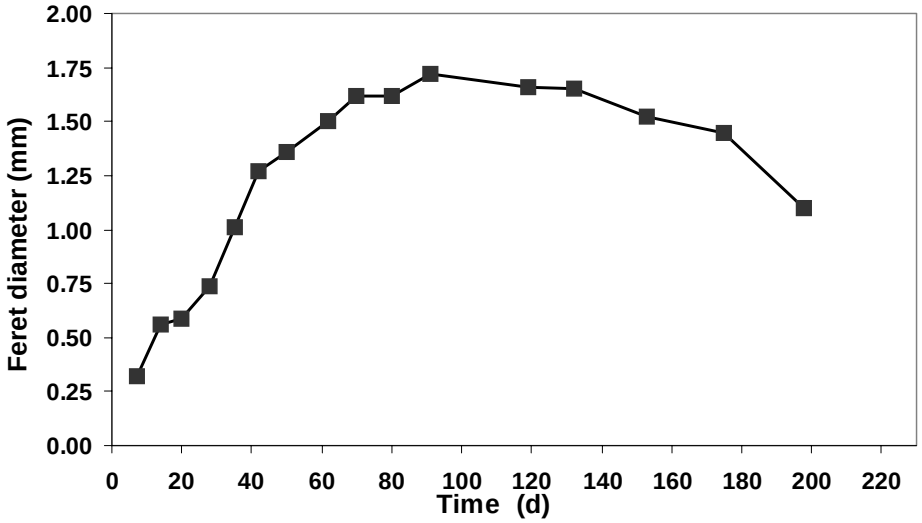


Figure 5.5. Distribution of average feret diameters along of the operation time.

During the whole operation time the cycle distribution in the reactor was similar to the one used by Beun *et al.* (1999). Biomass concentration in the reactor was around 0.2 g VSS/L at the beginning of the experiments (Figure 5.6). It increased up to 3 g VSS/L after 45 days, and then reached a stable value around 5-6 g VSS/L. However, at the end of the experiment and as consequence of a decrease of the TOC concentration the VSS also decreased up to 3 g VSS/L. From the slope of this figure it can be calculated the biomass yield, which was of 0.33 g VSS/g COD, similar to those found by Tay *et al.* (2001a) operating a granular reactor and slower than those reported for activated sludge of 0.42 g VSS/ g COD (Garrido *et al*., 2001).

The TSS concentration in the effluent was comprehended during most of the experimental period between 0.04 and 0.09 g TSS/L and the VSS were between 0.03 and 0.07 g VSS/L. However, at the beginning of the experiment and as a consequence of the wash-out of the sludge used as inoculum, the solids concentration in the effluent increased up to 0.20 g TSS/L and to 0.15 g VSS/L. After the operation day 190, the concentration of solids also increased up to 0.30 g TSS/L presumably as a result of the decrease of the TOC concentration applied to the system which could provoke the breaking up of the granules. The biomass concentrations obtained during this study were similar to those obtained in others granulars SBRs, between 3 and 7 g TSS/L, and also the VSS/TSS ratio, from 0.8 to 0.9 g/g (Arrojo *et al*., 2004).

The Sludge Volumetric Index (SVI) of the sludge in the reactor was around 30 - 40 mL/(g VSS) during most of the steady-state period (Figure 5.7). Nevertheless, at the end of the experiment it decreased slightly to 25 mL/(g VSS). The Zone Settling Velocity (ZSV) was in the range 8-14 m/h during the most operational time. Nevertheless, this value decreased up to 4 m/h at the beginning and at the end of the experiment due to the worsening of the settling properties of the sludge in these periods.

Biomass density was in the range from 25 to 45 g VSS/(L-granules) during most of the experimental period (Figure 5.8). This value was similar to the value reported for aerobic granules or biofilms formed in airlift reactors of 25-60 g VSS/($L_{granules}$) ((Kwok *et al.*, 1998) and higher than those reported by Beun (Beun

et al., 1999) of 11.9 g VSS/($L_{granules}$), 10 - 15 g VSS/($L_{granules}$) (Arrojo *et al*., 2004; Mosquera-Corral *et al*., 2005a) and 15-20 g VSS/($L_{granules}$) (Tijhuis *et al*., 1994).

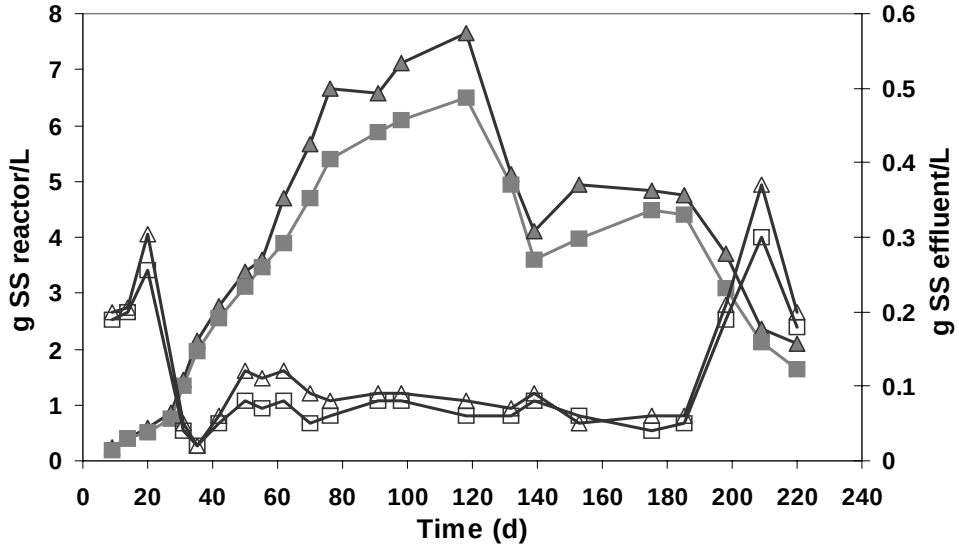


Figure 5.6. Concentrations of TSS (▲), VSS (■) in the reactor and TSS (Δ), VSS (□) in the effluent (right axis).

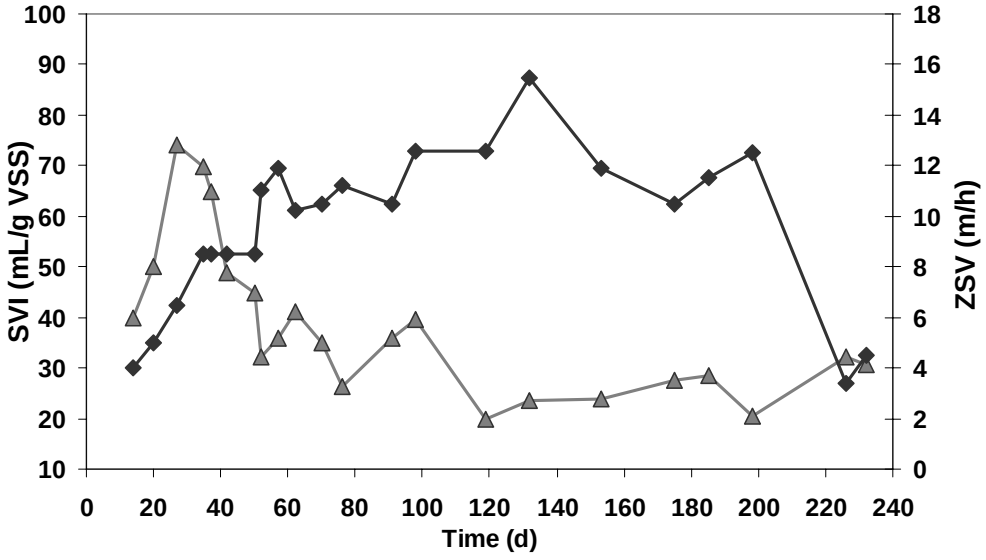


Figure 5.7. Evolution of the SVI (▲; left axis) and ZSV (◆; right axis) in the reactor.

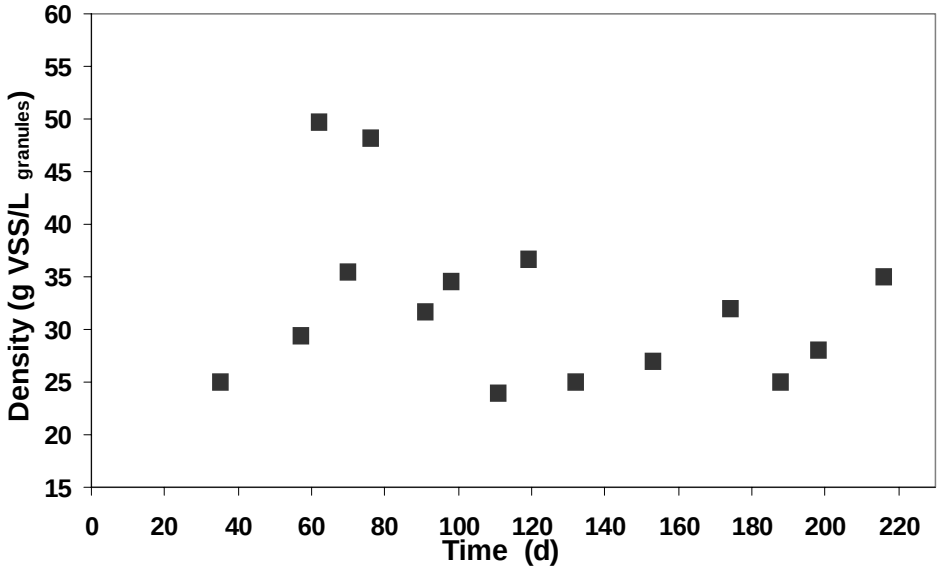


Figure 5.8. Evolution of biomass density in the reactor.

Microbiological analysis to identify the microbial populations were performed on samples of granules collected from the reactor and mechanically disrupted to be analysed by the FISH technique

Different samples of granules were collected in operating days 21, 35 and 58 (Figure 5.9) and targeted with several FISH probes in order to determine the main populations of microorganisms present. The probes used were Alf1b with the dye FITC for detection of *Alphaproteobacteria*, some *Deltaproteobacteria* and *Spirochaetes* and probe Gam42a with the dye Cy3 for detection of *Gammaproteobacteria* (Fig. 5.9A). In Figure 5.9B, the probe used was Ntspa712 with the dye FITC for detection of most member of phylum *Nitrospirae* (NOB) (Table 2.2, Chapter 2). DAPI was also applied, which dye of blue the entire DNA, so it can be inferred that there was a low quantity of bacteria belonging to the phylum *Nitrospirae*, according to the results obtained (Fig. 5.9B) where conversion to nitrate is neglected.

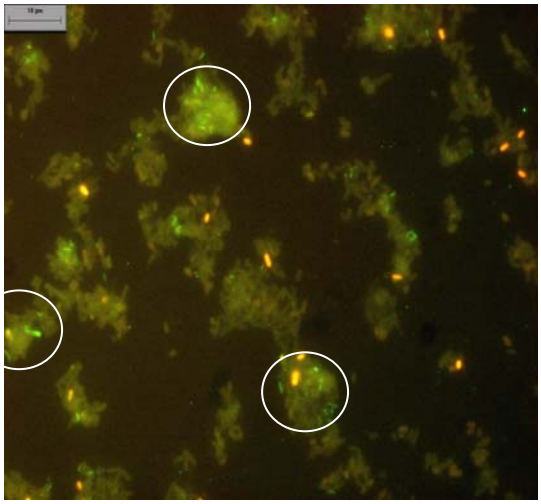
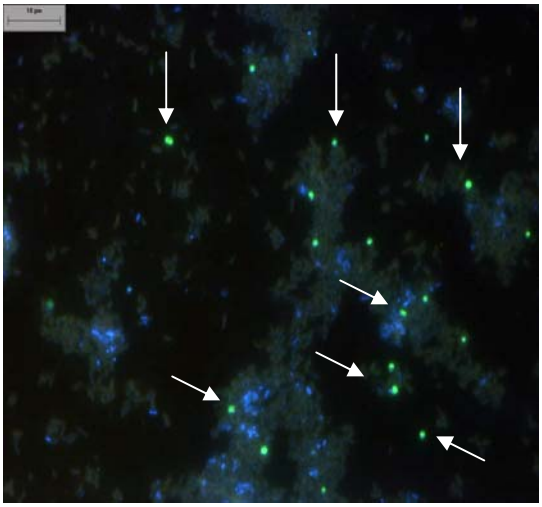
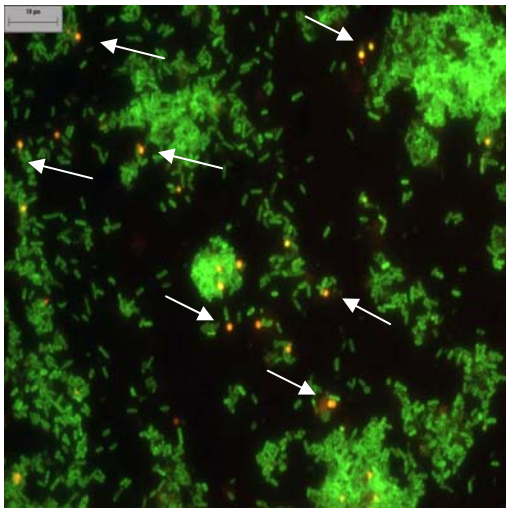
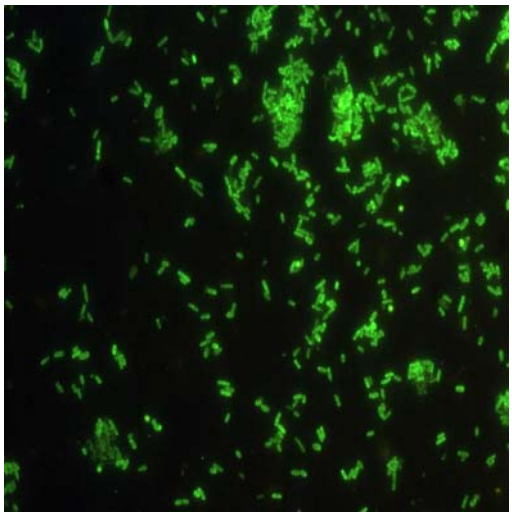
In the Figure 5.9C and 5.9D can be observed the different shape of the bacteria detected (probe EUB338) with rod-shape and cocci-shape. In red gave a

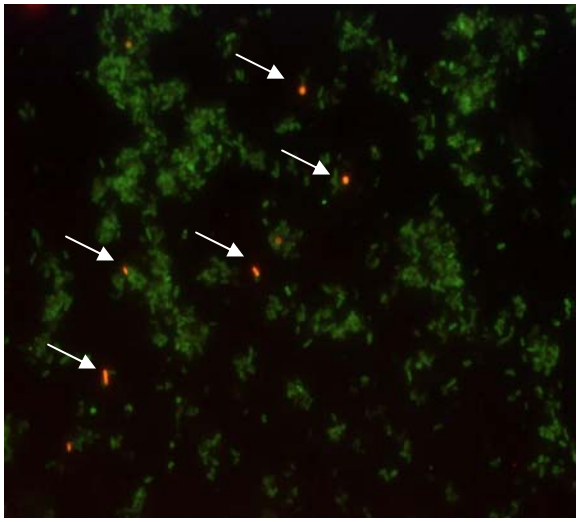
signal *Nitrosomonas spp* and, *Nitrosococcus mobilis* (AOB) (probe Nsm156) indicating the low presence of these ammonia oxidizing bacteria. The same probes that in Fig 5.9C were applied in Figure 5.9E for operating day 58.

In the Figure 5.9F, were detected with the dye FITC bacteria belonging to class *Alphaproteobacteria,* some to *Deltaproteobacteria* and *Spirochaetes* (probe Alf1b). Probe Bet42a was applied (dye Cy3 in red) simultaneously with DAPI, to show the amount of bacteria belonging to class *Betaproteobacteria* (Fig. 5.9G).

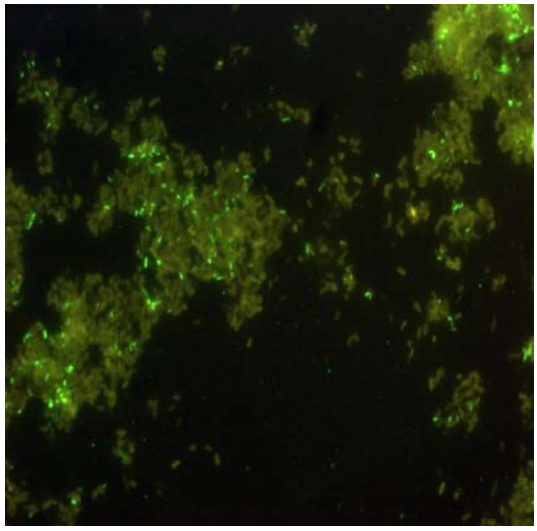
From the obtained experimental results it can be concluded thah nitrification didn´t occur during the operation of the reactor. However, some signal of ammonia oxidizing bacteria were detected. Schmid *et al*.(2005) found that for betaproteobacterial ammonia oxidizing bacteria, it has been shown thah ribosome content does not decrease significantly durng periods of starvation (Morgenroth *et al*., 2000) or inhibition (Schmid *et al*., 2001; Wagner *et al*., 2005). This property is most likely linked to their rigid and specialistic obligate chemolithotrophic way of life, which includes extreme resistante to starvation. Thus, the cellular rRNA content does not reflect the physiological activity of these organisms.

Other parameter which influenced the no presence of nitrification could be the cellular retention time, which was always lower than 20 days. This value was too low to favour the nitrification bacteria growth which have a slow growth rate.

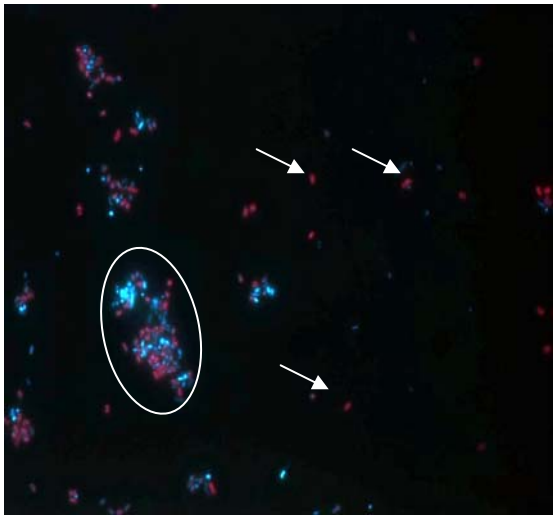
	
A) *Alphaproteobacteria*, some *Deltaproteobacteria* and *Spirochaetes* (Alf1b; green) and *Gammaproteobacteria* (Gam42a; red). Operating day: 21	**B) Ntspa712 (NOB; green) and DAPI (blue)** **Operating day: 35**
	
C) EUB338 (green) and Nsm156 (AOB; red) **Operating day: 35**	**D) EUB338** **Operating day: 35**



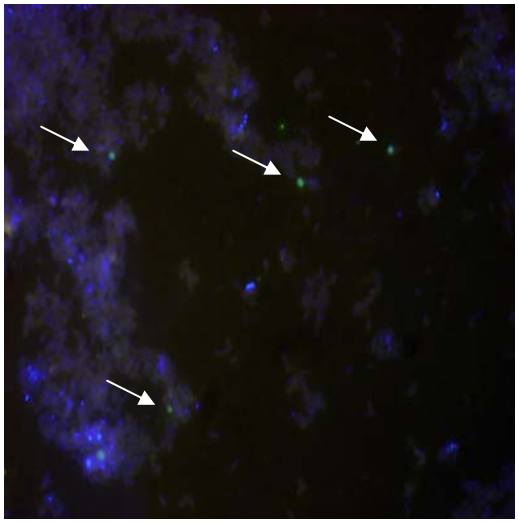
E) Eub338 (green) and Nsm156 (AOB; red)
Operating day: 58



F) Alf1b
Operating day: 58



G) Bet42a (in red) and DAPI
Operating day: 58



H) Ntspa712 (NOB; green) and DAPI
Operating day: 58

Figure 5.9. FISH images of the biomass in the granules in different operating days.

5.4.2. Carbon and nitrogen removal in the system

The reactor was fed with acetate as sole carbon source and ammonium as N source. During the first stage the applied organic loading rate (OLR) and nitrogen loading rate (NLR) were of 2 g COD/(L·d) and 0.1 g NH_4^+-N/(L·d), respectively. The reactor was operated with a TOC/N ratio of 7.5 g/g during 120 days. The TOC concentration in the influent was around 200 mg/L being in the effluent lower than 30 mg/L (Figure 5.10).The ammonia concentration in the influent was 25 mg N/L and the NLR of 0.1 g N/L·d (Figure 5.11). The main process for nitrogen removal in the SBR was the nitrogen assimilation for biomass growth and nitrification did not occur. The averaged biomass concentration assimilated was calculated considering a general composition of the biomass as $C_5H_7NO_2$ and using the same equations shown in Chapter 4. From this nitrogen balance, the 90% of the nitrogen removal was nitrogen for biomass growth.

During the following periods (Period II and III), the concentration of organic matter was progressively decreased keeping the concentration ammonium constant of 25 mg/L and later (Period IV) this concentration was increased until 50 mg/L (Table 5.3). During period II the TOC/N ratio applied was 3 g/g. The TOC concentration in the influent was decreased to 75 mg N/L and concentrations in the effluent were around 12 mg NH_4^+-N/L, 1-3 mg NO_3^--N/L and 0 mg NO_2^--N /L. The OLR applied to the reactor was of 0.8 g COD/(L·d). During Period III the OLR applied was of 0.4 g COD/(L·d) and the fed TOC/N ratio was 1.5 g/g. However, no changes were observed in the effluent composition. During the last period the ammonium concentration in the influent was doubled operating at TOC/N ratio of 0.75 g/g but nitrification was not observed. After a few days operating at TOC/N ratio of 0.75 the formation of small aggregates with an average feret diameter around 0.90 mm was observed.

Overall TOC removal efficiency was, during the whole operational time, between 80 and 95%. Lower values than those and around 70 % were measured at occasional stages (Figure 5.12). N removal efficiency was around 20-40% during the whole operational time.

The low TOC concentration in the influent during period IV, of only 0.4 g COD/(L·d) led to granule instability and biomass washout. During this period biomass had a fluffy, irregular and loose morphology (Figure 5.13). Also small particles were observed in this period probably coming from the breakage of the big original granules.

These results were similar to those found by de Kreuk (de Kreuk *et al.*, 2006) who demonstrated that granule formation with domestic sewage was only possible if the reactor was operated at OLR higher than 1.6 g COD/(L·d). They found that COD load is crucial during startup, which might be hampered if the sewage is too diluted or cycle time are too long. Similar results were observed by Tay *et al.* (2003), who studied the influence of organic loading rate on the formation of aerobic granules. They didn´t obtain granules formed under the OLR of 1 g COD/(L·d). So, COD load will be an important process parameter at larger scale operation and should be taking into account.

However, in a previous work (Chapter 4) Mosquera-Corral *et al.* (2005) obtained a nitrifying granular sludge from heterotrophic sludge by decreasing the COD/N until COD was completely eliminated. In spite of the changes in the feeding composition the granules maintained their structures and the solids content in the effluent was reduced to 10 mg TSS/L when acetate was removed from the feeding media.

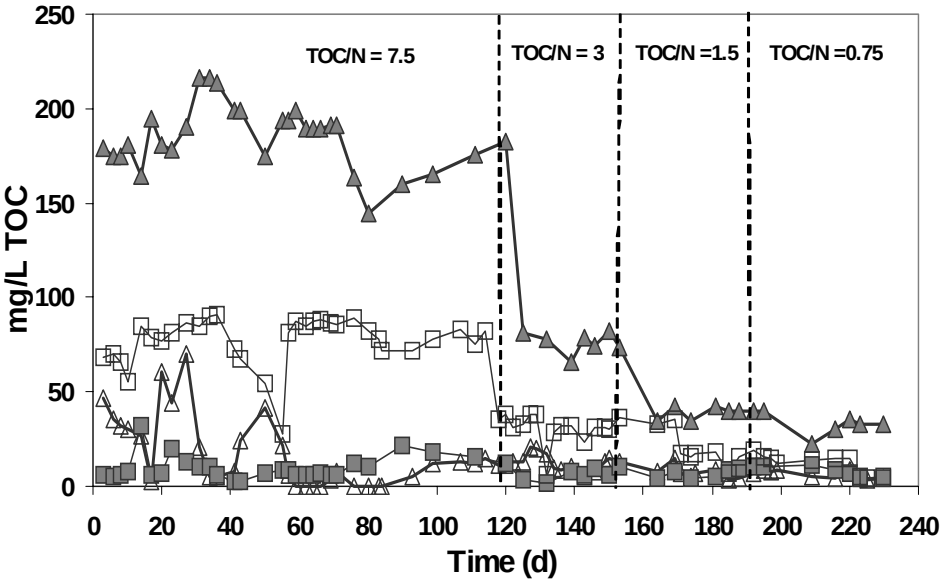


Figure 5.10. Total organic carbon in the influent (▲) and in the effluent (△), inorganic carbon in the influent (■) and in the effluent (□).

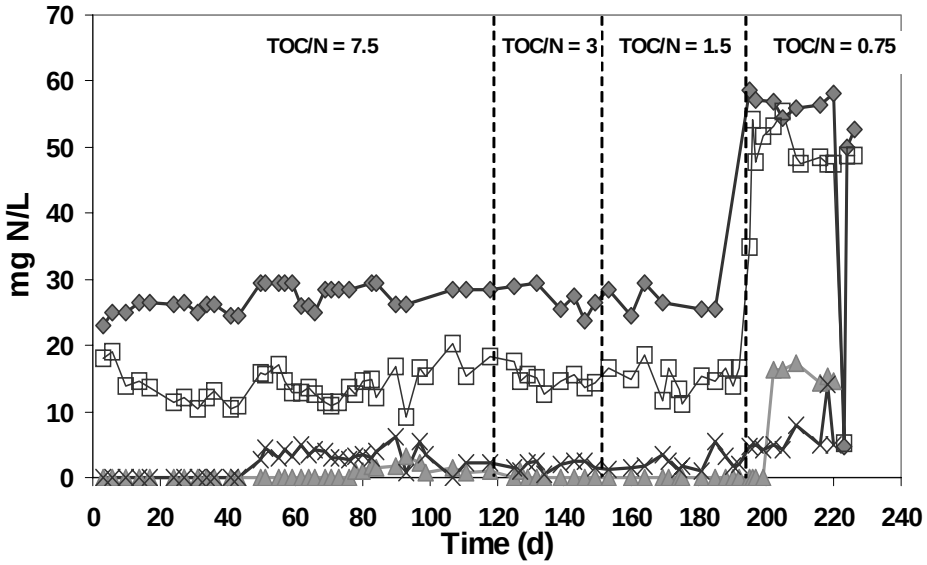


Figure 5.11. Ammonia concentration in the influent (♦) and in the effluent (□), nitrate (×) and nitrite (▲) concentration in the effluent.

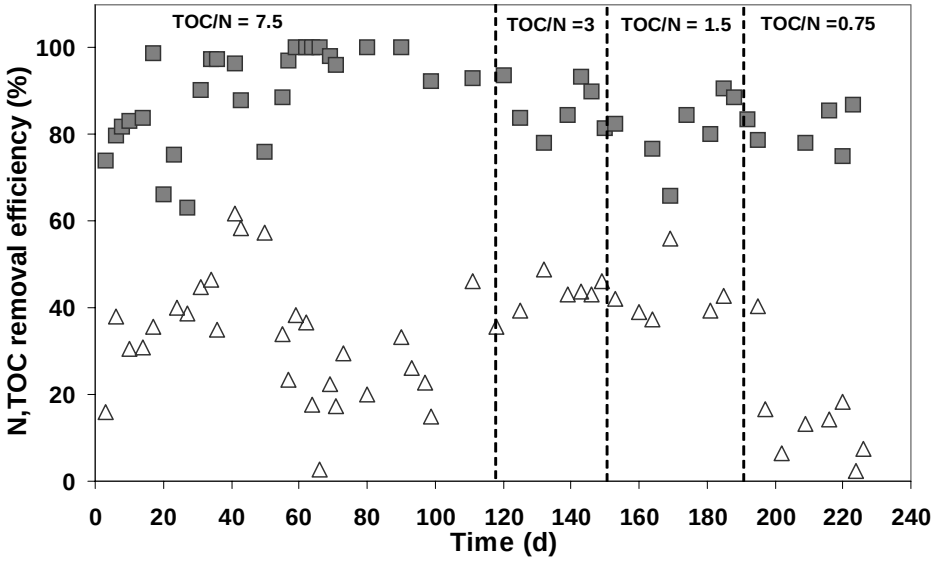


Figure 5.12. N (△) and TOC (■) removal efficiency.

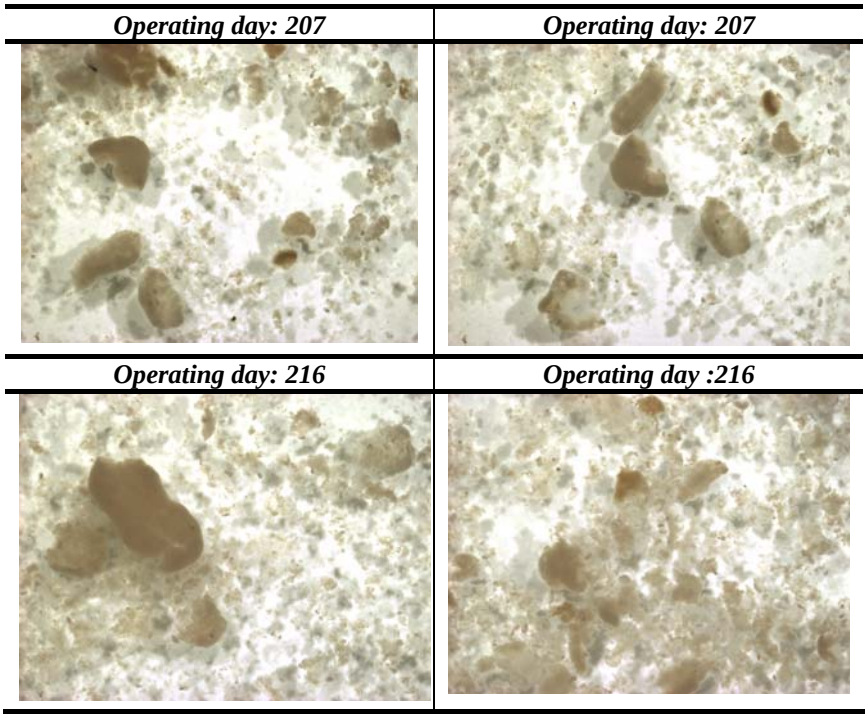
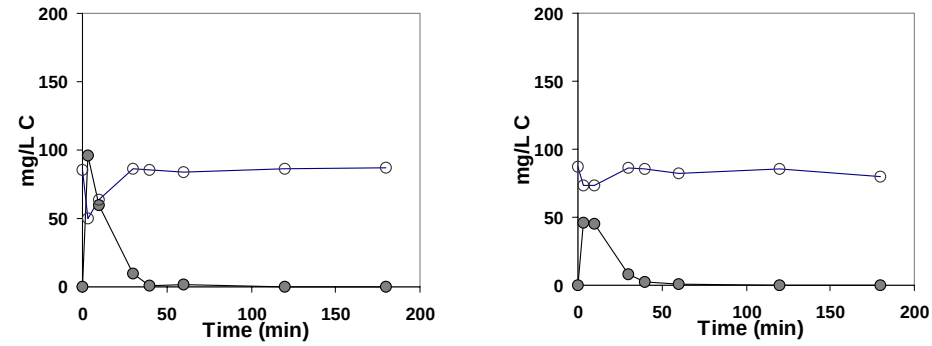


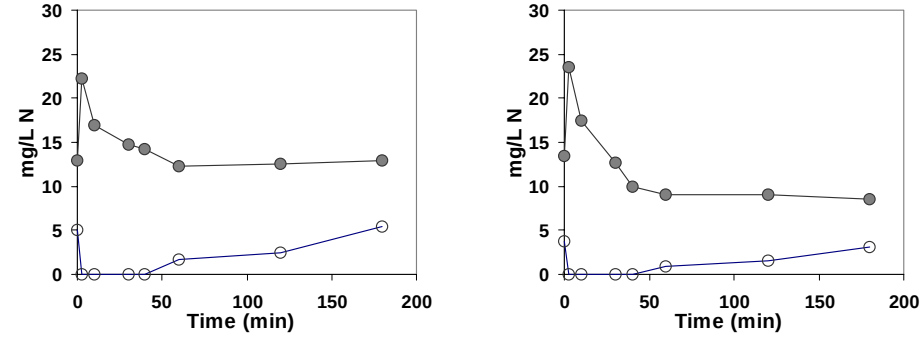
Figure 5.13. Granules broke in pieces during period IV.

The measurement of the concentrations of these compounds (carbon and nitrogen) was performed periodically along several operational cycles in the different operational periods (Figure 5.14, 5.15).

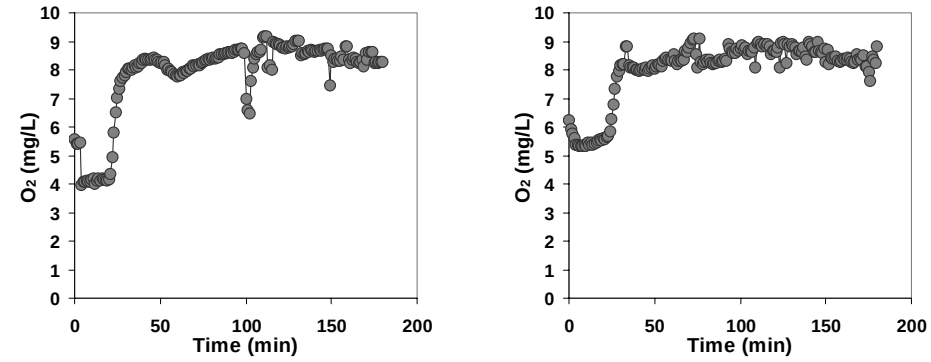
Figure 5.14 shows profiles measured on operating day 62, when the reactor was operated with TOC/N ratio of 7.5 g/g, and on operating day 140 operating the reactor with TOC/N ratio of 3.0 g/g. In both operating days, no nitrification or denitrification process were carried out. The DO concentration was of 4 mg O_2/L during first minutes of the cycle, and increased up to 8-9 mg O_2/L during the rest of the cycle. Almost all the TOC disappeared completely during the first 10 minutes of the cycle. The TOC concentrations measured at the end of the cycle were similar in both operating days and it was practically consumed and partly stored in the biomass. Ammonia was partly oxidised to nitrate (but only around 5 mg/L NO_3^--N were reached) during the aerobic period immediately after the disappearance of TOC from the liquid phase. Figure 5.15 shows profiles measured on operating days 175 and 209 when the TOC/N ratio was decreased in order to favour the nitrification but however no nitrification was reached during these periods because of the breakage of the granules.



A)



B)



C)

Figure 5.14. Typical concentration profiles during a cycle of the SBR on day 62 (Period I) and on day 140 (Period II). A) (•) TOC; (o) IC, . B) (•) N-NH_4^+; (o) N-NO_3^-. C) Dissolved oxygen concentration

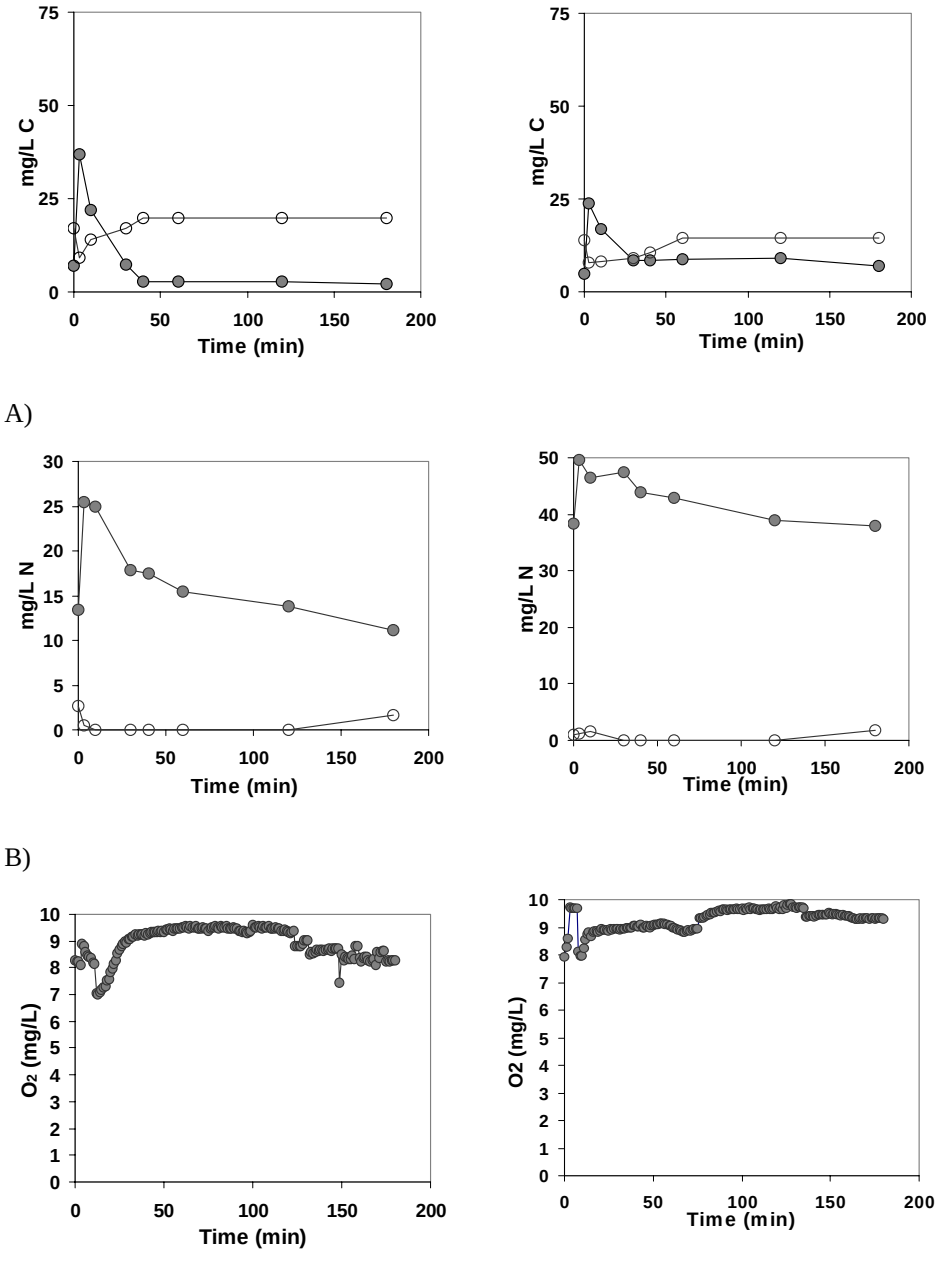
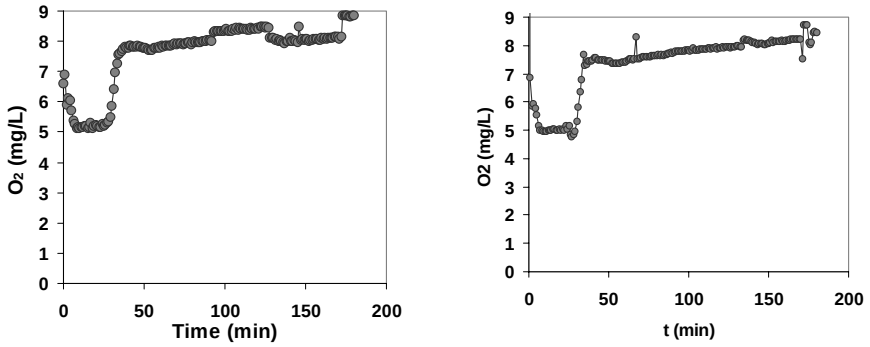


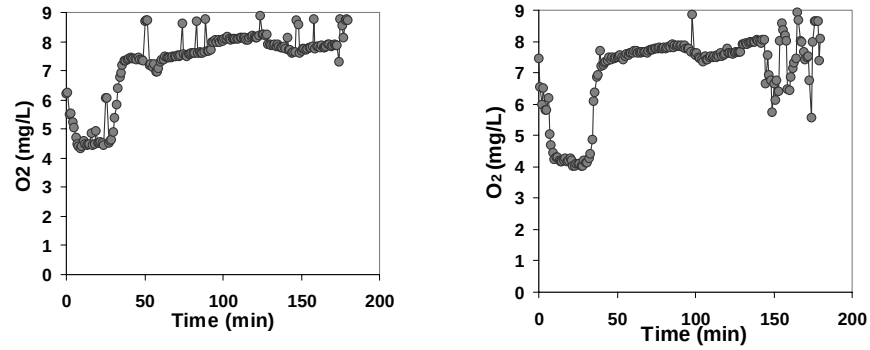
Figure 5.15. Typical concentration profiles during a cycle of the SBR on day 175 (Period III) and on day 209 (Period IV). A) (•) TOC; (o) IC, . B) (•) N-NH_4^+; (o) N-NO_3^-. C) Dissolved oxygen concentration

5.4.3. Effect of mechanical stirrer

The effect of operating hydrodynamic conditions on the granulation was studied in the SBR where complete mixture was achieved by means of mechanical stirring and air flow. Figure 5.16 shows the profiles of the dissolved oxygen (DO) concentration on different operating days (60 and 70) using the mechanical stirrer (A) and without stirrer (B). From these profiles it can be concluded that the mechanical stirrer almost had no significant effect on the DO concentration since a high upflow velocity is needed in order to maintain high oxygen concentration in the medium. In this study, the reactor was operated with an upflow velocity of 1.58 cm/s similar to those used by Tay *et al.* (2001b).



A)



B)

Figure 5.16. Dissolved oxygen concentration on operating days 60 and 70 . A) With mechanical stirrer and B) without mechanical stirrer.

As far as DO is concerned, de Kreuk *et al.* (2004) and Mosquera-Corral *et al.* (2005b) reported that aerobic granules with heterotrophic bacteria were not stable if DO decreased to 40% of saturation. As a common approach in evaluating shear rate in bubble column involves assuming that an average shear rate exists in the column and proportional to superficial gas velocity (Al-Marsy, 1999), the decrease of aeration rate also results in the decrease of hydrodynamic stress.

Although aerobic granular biomass has been achieved mainly in reactors where mixture is achieved by gas flow they can be feasibly produced in CSTR systems.

5.5. Conclusions

- Granulation in aerobic conditions has been intentionally produced in reactor with mixture induced by mechanical stirring and superficial upflow air velocity of 1.58 cm/s.
- The formation of granules in the SBR was achieved by using a reactor with an unusual geometry meaning that the H/D ratio of 2.5. Granules with good settling properties were obtained, SVI of 30-40 mL/g VSS, and ZSV higher than 8 m/h. This made feasible to operate the system with high exchange volume and thus organic and nitrogen loading rates applied to the system were up to 2 g COD/(L·d) and 0.1 g NH_4^+-N/(L·d).
- The operation of the aerobic granular SBR at different TOC/N ratios allowed the achievement of removal percentages of 90% for the organic matter and up to 40% for the ammonia nitrogen.
- The formation of stable granules was not possible for OLR under 1 g COD/(L·d) meaning that a minimum gradient concentration is needed to be able to generate granular biomass.
- The TSS concentration in the effluent was between 40 and 90 mg TSS/L during the different operational stages except for the last one where TSS increased to 0.3 g TSS/L due to the breakage of the granules.

5.6. References

Al-Marsy, W.A. (1999). Effect of scale-up on average shear rates for aerated non–Newtonian liquids in external loop airlift reactors. *Biotechnology and Bioengineering*, **62**, 494-498.

Amann R.I. (1995). *In situ* identification of micro-organisms by whole-cell hybridization with r RNA-targeted nucleic acid probes, p.1-15. In A.D.L: Akkerman, J.D. van Elsas, and F.J. de Brujin (ed.), *Molecular microbial ecology manual*. Kluwer Academic Publisher, Dordrecht, The Netherlands.

APHA-AWWA-WPCF. (1999). Standard Methods for the examination of water and wastewater. 20th Edition. Clesceri, L.S., Greenberg, A.E. & Eaton, A.D. (eds.).

Arrojo B., Mosquera-Corral A., Garrido J.M. and Méndez R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research* **38**, 3389-3399.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P:A: and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research,* **33** (10), 2283-2290.

Beun J.J., van Loosdrecht M.C.M. and Heijnen J.J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research*, **36**, 702-712.

Chang H.T., Rittmann B.E., Amar D.R., Ehrlinger O. and lesty Y. (1991). Biofilm detachment mechanisms in a liquid fluidized bed. *Biotechnology and Bioengineering*, **38**, 499-506.

de Bruin L.M.M., de Kreuk M.K., van der Roest H.F.R., Uijterlinde C. and van Loosdrecht M.C.M. (2004). Aerobic granular sludge technology: and alternative to activated sludge. *Water Science and Technology*, **49** (11-12),1-7.

de Kreuk M.K. and van Loosdrecht M.C.M. (2004). Selection of slow growing organisms as a means for improving aerobic granular sludge stability. *Water Science and Technology*, **49**, 9-17.

de Kreuk M.K. and van Loosdrecht M.C.M. (2006). Formation of aerobic granules with domestic sewage. *Journal of Environmental Engineering*, **132** (6), 694-697.

Garrido J. M., Omil F., Arrojo B., Méndez R.. and Lema J. M**.** (2001). Carbon and nitrogen removal from a wastewater of an industrial dairy laboratory with a coupled anaerobic filter-sequencing batch reactor system. *Water Science and Technology*, **43**, 315–321.

Gjaltema A., van Loosdrecht M.C.M. and Heijnen J.J. (1997). Abrasion of suspension biofilm pellets in airlift reactors: effect of particle size. *Biotechnology and Bioengineering*, **55**, 206-215.

Jiménez B., Noyola A., Capdeville V., Roustan M. and Faup G. (1988) Dextran Blue coulorant as a reliable tracer in submerged filters. *Water Research*, **22**, 1253-1257.

Kwok W.K., Picioreanu C., Ong S.L., van Loosdrecht M.C. M., Ng W.j. and Heijnen J.J. (1998). Influence of biomass production and detachment forces on biofilm structures in a biofilm airlif suspension reactor. *Biotechnology and Bioengineering*, **58**, 400-407.

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

Liu Y.Q., Liu Y. and Tay J.H. (2004). The effects of extracellular polymeric substances on the formation and stability of biogranules. *Applied Microbiology and Biotechnology*, **65**, 143-148.

Liu Y. and Liu, Q.-S. (2006). Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. *Biotechnology Advances*, **24**(1), 115-127.

Liu Y-Q. and Tay J-H. (2006). Variable aeration in sequencing batch reactor with aerobic granular sludge. *Journal of Biotechnology*, **124**, 338-346.

Morgenroth E., Obermayer A., Arnold E., Brühl A., Wagner M. and Wilderer P.A. (2000). Effect of long-term idle periods on the performance of sequencing batch reactors. *Water Science and Technology*, **41**, 105-113.

Mosquera-Corral A., Vázquez-Padín J.R., Arrojo B., Campos J.L. and Méndez R. (2005a). Nitrifying granular sludge in a sequencing batch reactor. *In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing.* Munich, 63-70.

Mosquera-Corral A., de Kreuk M.K., Heijnen J.J. and van Loosdrecht M.C.M. (2005b). Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. *Water Research*, **39** (12), 2676-2686.

Moy B.Y.P., Tay J.H., Toh S.K. Liu Y. and Tay S.T.L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Letters in Applied Microbiology*, **34,** 407-412.

Smolders G. J. F., Klop J., van Loosdrecht M.C. M. and Heijnen J.J. (1995). A metabolic model of the biological phosphorus removal process. Effect of the sludge retention time. *Biotechnology and Bioeng*ineering, **48**, 222-233.

Schmid M., Schmitz-Esser S., Jetten M. and Wagner M. (2001).16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental Microbiology*, **3**, 450-459.

Schmid M, Mass B., Dapena A., et al. (2005). Biomarkers for In situ Detection of Anaerobic Ammonium-Oxidizing (Anammox) bacteria. Minireview. *Applied and Environmental Microbiology*, **71** (4), 1677-1684.

Shin H. S., Lim K.H. and Park H. S. (1992). Effect of shear stress on granulation in oxygen aerobic upflow sludge reactors. *Water Science and Technology*, **26**, 601-605.

Soto M., Veiga M.C., Méndez R. and Lema J.M. (1989). Semi-micro COD determination method for high salinity wastewater. *Environmental Technology Letters*, **10**(5), 541-548.

Tay J-H, Liu Q-S and Liu Y. (2001a). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology* **33**, 222-226.

Tay J-H, Liu Q-S and Liu Y. (2001b). The effects of shear force on the formation, structure and metabolism of aerobic granules. *Applied Microbiology and Biotechnology*, **57**, 227-233.

Tay J-H, Liu Q-S and Liu Y. (2002a). Aerobic granulation in sequential sludge blanket reactor. *Water Science and Technology*, **46** (4-5), 13-18.

Tay J-H, Liu Q-S and Liu Y. (2002b). Hydraulic slection pressure-induced nitrifying granulation in sequencing batch reactors. *Applied Microbiology Biotechnology*, **59**, 332-337.

Tay J-H, Pan S., Tay S.T.L., Ivanov V. and Liu Y. (2003). The effect of organic loading rate on the aerobic granulation: the development of shear force theory. *Water Science and Technology*, **47** (11), 235-240.

Tay J.-H., Liu Q.-S. and Liu Y. (2004). The effect of upflow air velocity on the structure of aerobic granules cultivated in a sequencing batch reactor. *Water Science and Technology*, **49**, 11-12, 35-40.

Tay J-H., Tay T-L., Liu Y. and Ivanov (2006). Biogranulation technologies for wastewater treatment. *Waste management series*.

Tijhuis L., van Loosdrecht M.C.M. and Heijenen J.J.(1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioeng*ineering, **44**, 595-608.

Trinet F., Heim R., Amar D. Chang H.T. and Ritmann B.E. (1991). Study of biofilm and fluidization of bioparticles in a three-phase liquid-fluidized-bed reactor. *Water Science and Technology*, **23**, 1347-1354.

van Benthum W.A.J., Van Loosdrecht M.C.M. and Heijnen J.J. (1997). Control of heterotrophic layer formation on nitrifying biofilms in a biofilm airlift suspension reactor. *Biotechnology and Bioengineering*, **53**, 397-405.

van Loosdrecht M.C.M., Tijhuis L., Widieks A.M.S. and Heijnen J.J (1995). Population distribution in aerobic biofilms on small suspended particles. *Water Science and Technology* **31**, 163–171.

Wagner M., Rath G., Amann R., Koops H.-P. and Schleifer K.-H. (1995). In situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology*, **18**, 251-264.

Wang F., Liu Y.-H., Yang F.L., Zhang X.W. and Zhang H.M. (2005). Study on the stability of aerobic granules in a SBAR- effect of the superficial upflow air velocity and carbon source. In: Aerobic Granular Sludge. Water and Environmental Management Series. IWA Publishing. Munich, 35-42.

Chapter 6

Effects of hydrodynamic conditions on the performance of Anammox granular Sequencing Batch Reactor (SBR)[1, 2]

Summary

The effect of operating hydrodynamic conditions on the Anammox process was studied in sequencing batch reactor (SBR) where complete mixture was achieved by means of mechanical stirring (SBRM) or gas flow (SBRF1 and SBRF2).

The reactor SBRM was operated during 218 days under different stirring speeds (60 - 250 rpm) and the reactors SBRF1 and 2 were operated for 140 and 110 days respectively under different upflow velocities (3.53 – 12.35 cm/min). In this way the reactors were exposed to different shear conditions and the stability and performance of the Anammox granules was studied.

The nitrogen loading rate (NLR) fed to the SBR ranged from 0.05 g N/(L·d) to 0.3 g N/(L·d), being the latter the chosen value during stable conditions. The nitrite (limiting substrate) removal percentage was 98% during most of the operational period.

The specific Anammox activity of the biomass was practically constant and around 0.4 g N/(g VSS·d) for the SBRM and 0.35 g N/(g VSS·d) for the SBRF2.

Part of this chapter has been published as:

[1] **Arrojo B., Mosquera-Corral A., Campos J.L. and Méndez R.** (2006). Effects of mechanical stress on Anammox granules in a Sequencing Batch Reactor (SBR). *Journal of Biotechnology*, **123**, 453-463.

[2] **Arrojo B., Figueroa M., Mosquera-Corral A., Campos J.L. and Méndez R.** (2006). Effects of hydrodynamic shear forces on Anammox granules. (*Submitted*).

The average feret diameter of the formed granules was 0.64 mm and 0.75 mm for the SBRM and SBRF1, 2, respectively.

Limit values for the accurate operation of the Anammox granular systems were around 180 rpm and 7.39 cm/min for the SBRM and SBRF respectively. In the SBRM the Anammox activity decreased to 50% when a rotating speed of 250 rpm was tested and the average diameter decreased in 45%, the concentration of solids in the effluent increased to 0.2 g TSS/L and nitrite was accumulated in the reactor up to 60 mg N/L. In the case of the SBRF the Anammox activity decreased to 85% when upflow velocity of 9.7 cm/min was applied and the average diameter decreased in a 30% while nitrite accumulated in the reactor up to 70 mg N/L.

6.1. Introduction

The Anammox process is an alternative to remove nitrogen compounds from high nitrogen loaded wastewater with low organic matter content, instead of the traditional combined nitrification/denitrification processes. This process consists of the anaerobic oxidation of ammonia using nitrite as electron acceptor according to the stoichiometry described by Strous *et al.* (1999) (equation [6.1]). This process allows a saving of oxygen supply and organic matter compared to nitrification/denitrification processes.

$$NH_4^+ + 1.31\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \quad [6.1]$$

Recently the number of research works focused on the study of the Anammox process has increased. Some of them applied to the study of the metabolic pathways of the process (Schmidt *et al*., 2002; Strous *et al*., 2002) or to the identification of Anammox microorganisms (Schmid *et al*., 2001; Mohan *et al*., 2004). Nevertheless, there are scarce studies related to the engineering aspects to implant this process at full scale.

The industrial application of the Anammox process is still difficult due to the slow growth rate of the Anammox micro-organisms which present doubling times in the range of 11 - 15 days (Strous *et al*., 1999; Dapena-Mora *et al*., 2004a). For this reason long starting up periods are needed to achieve stable operation conditions. Besides the Anammox process is inhibited by its substrates, especially nitrite (Strous *et al*., 1999; Guven *et al*., 2005). These two factors make it necessary the use of reactor systems which present good characteristics of biomass retention and complete mixture conditions. The Sequencing Batch Reactors (SBR) (Strous *et al*., 1999) and continuous gas-lift reactors (Dapena-Mora *et al*., 2004a; Sliekers *et al*., 2003) have been found to be appropriated to fulfil these conditions. Dapena-Mora *et al.* (2004a) went further and showed that the SBR is a suitable system to grow Anammox biomass in form of granular sludge. Gas lift reactors where Anammox biomass was in the form of aggregates were already successfully operated (Sliekers *et al.,* 2003; Dapena-Mora *et al*., 2004a). Sliekers *et al.* (2003) operated a gas-lift reactor with granular biomass

treating nitrogen removal rates of 8.9 kg-N/(m^3·d). The complete mixture was achieved by sparging the gas (Ar/CO_2 95%/5%) from the bottom of the reactor at a gas upflow of 200 mL/min of 7.8 cm/min.

Thus complete mixture conditions can be achieved by means of mechanical stirring (SBR) or gas recirculation (gas-lift). Both processes cause shear forces which can have several effects on the biomass. These forces play an important role on the formation of well settling biofilm particles in aerobic (Kwok *et al.*, 1998; Chang *et al*., 1991; Gjaltema *et al*., 1997; van Benthum, 1996, 1997; Liu and Tay, 2002; Tijhuis *et al*., 1994), anaerobic (Alphenaar *et al*., 1993; O'Flaherty *et al.*, 1997; Arcand *et al.*, 1994; Liu and Tay, 2003) and anoxic conditions (Klapwijk 1979, 1981). Therefore, it could be very positive to operate at relative high shear stress forces in the Anammox reactor to favour the granule compactness and to generate a stronger structure of biomass.

On the other hand an excessive high shear stress can possibly mean increasing the wash-out of the biomass and a consequent lost of activity as it was observed in ferementation systems (Chisti, 2000; Sánchez-Mirón *et al*., 2003).

When stable granular sludge is produced it is retained inside the reactor easier than the flocculent sludge due to its better settleability characteristics. Since the Anammox biomass can grow forming granules it is important to have favourable conditions for granulation in order to obtain a stable Anammox population.

The shear force resulting from hydraulics and/or particle-particle collision is a key factor that influences the formation, structure and stability of biomass aggregates in aerobic conditions (Kwok *et al.*, 1998; Liu and Tay, 2002). However contradictory results have been found in the literature regarding the effects of the shear stress due to stirring over the granular formation and stability. On one hand, the application of high shear forces would result in a strong and homogeneous biofilm formation in aerobic conditions (Chang *et al.*, 1991; Chen *et al.*, 1998; Kwok *et al*., 1998; Gjaltema *et al*., 1997). This observation was supported by Tay *et al.* (2001) who observed that the shear force exerts positive effects on the production of polysaccharide compounds which are supposed to act as biological glue and favour the formation of a stable granular structure. These authors also demonstrated that shear stress at certain levels plays a crucial role in

aerobic granulation and influences the structure and metabolism of granules. In case of anaerobic sludge the formation, structure and metabolism of immobilized microbial community are very closely associated with hydrodynamic shear force in reactors. In an anaerobic culture to promote bacteria to form granules a number of conditions have to be fulfilled in the way that the contributions of physical, chemical and biological forces to granulation process could not be considered separately (Liu *et al*., 2003). However, the mechanisms how hydrodynamic shear forces influence the formation, structure and metabolism of biofilms and granular sludge are not yet understood. On the other hand, what is clear is the existence of a limit value for the shear stress which causes partial loss of biomass activity and decrease of the size of the biomass granules (Chisti, 2000). Taking into account this information it seems logical to state that there is a maximum mechanical stress allowed for the optimum performance of the Anammox processes in SBR systems. The minimum mechanical stress is limited by the achievement of the complete mixture inside the system and for the fact that shear forces play an important role on the formation of well settling biofilm particles (Tijhuis *et al*., 1996, Kwok *et al.,* 1998; van Benthum *et al*., 1996; Noyola and Moreno, 1994). Therefore, it could be very positive to increase the shear stress in the Anammox reactor to favour the granule compactness and the stronger structure of biomass. But an excessive high shear stress means increasing the wash-out of the biomass and a lost of activity.

6.2. Objectives

The aim of this work was the study of the effects of the mechanical stress and gas recirculation on the stability of the Anammox granular biomass and the efficiency of the process. The shear forces were modified by the stepwise increase of the stirring speed and the gas upflow velocity in a CSTR and a gas bubble/gaslift reactor, respectively. The stability of the Anammox granular biomass was studied in terms of biomass activity, density, settling properties, and diameter and size distribution of the granules. The efficiency of the process was checked in terms of nitrogen removal.

6.3. Materials and methods

6.3.1. Experimental set-up

Three reactors were used in this work:

- SBRM: A completely stirred tank reactor provided by an impeller for complete mixture.
- SBRF1: A bubble column reactor mixed with gas flow.
- SBRF2: A gas lift reactor provided with an inner tube (riser) and mixed by means of gas flow.

Completely Stirred Tank Reactor (SBRM)

The completely stirred tank reactor was provided with a working volume (V) of 1 L, a diameter (D) of 0.1 m and a height/diameter (H/D) ratio of 1.5 (Figure 6.1A). The mixture inside the reactor was achieved by means of a mechanical stirrer operated at rotating speeds ranging from 60 to 250 rpm.

The used mechanical stirrer was a Rushton turbine (Figure 6.1B), with a standard 6-blade disk impeller. The Rushton turbine had an impeller diameter (d) of 0.06 m with a blade width (w) of 0.02 m and an impeller height (h) of 0.015 m. The impeller diameter to reactor diameter (d/D) ratio was 0.60. The stirrer length (l) was 0.07 m with an area of impeller (a) blades of 0.0018 m^2.

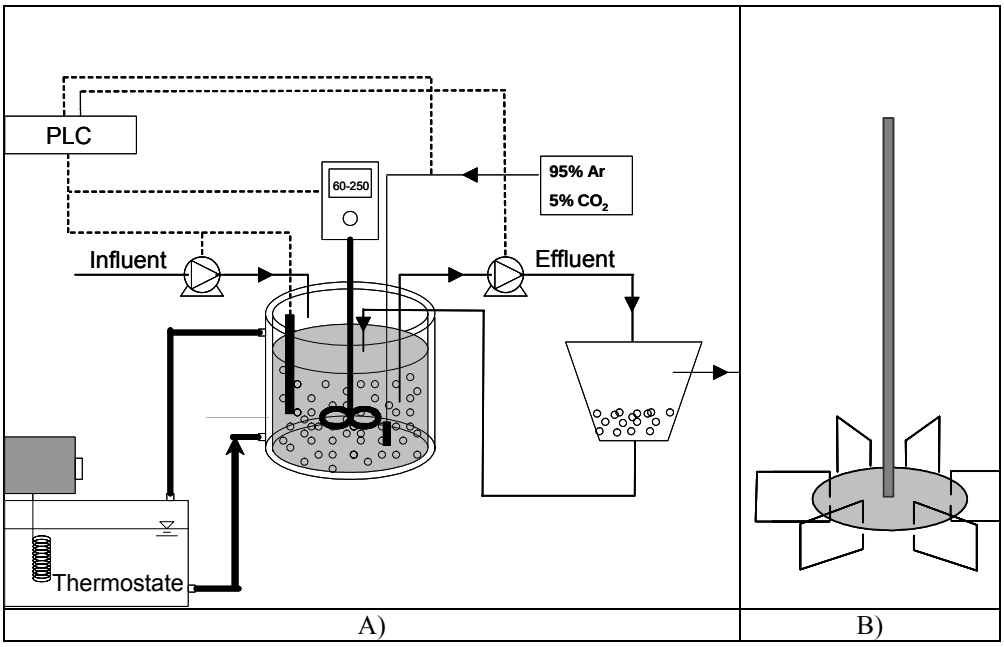


Figure 6.1. A) Experimental set-up of the sequential batch reactor (SBR); B) Rushton impeller.

Bubble column (SBRF1) and gas lift reactor (SBRF2)

Both reactors presented a total volume of 2.5 L and a working volume of 1.5 L was used. Dimensions of the units were: H = 465 mm, D = 85 mm, H/D = 5.5 (Figure 6.2). The maximum level of the liquid was 264 mm, and the minimum level after effluent withdrawal was 132 mm. The mixture inside the reactor was achieved by means of the off-gas recirculation.

The gas lift reactor contained an inner tube (the riser) concentrically placed in the reactor in order to create enough turbulence to generate a circular flow of biomass and liquid. The riser diameter to reactor diameter ratio was of 0.71. Dimensions of the riser are detailed in Table 6.1.

Table 6.1. Dimensions of the riser.

Parameter	*Value*
Riser height (mm)	200
Riser diameter (mm)	60

In the three reactors a set of two peristaltic pumps was used to feed and to discharge the effluent, respectively. The influent was introduced in the system through ports located at the top of the reactors. The effluent was discharged through the sampling port placed at middle height of the reactor. Norprene tubing and connections were used to avoid oxygen leakage into the reactors. The SBR temperature was fixed at 30 ± 1 ºC by means of a thermostated jacket. The operational pH ranged between 7.5 and 8.0 without control. The reactors were flushed with a mixture of 95% Ar and 5% CO_2 to maintain anaerobic conditions.

The three reactors were operated as sequencing batch reactors (SBR) in cycles of 6 hours (Dapena-Mora *et al.,* 2004b). Control of the system was done with a PLC (CPU224, Siemens). Each cycle comprised four phases: during the first phase the reactor was continuously fed in complete mixed conditions during 300 minutes; in the second phase the feeding was stopped during 30 minutes maintaining the stirring; the third phase of settling lasted 20 minutes; and finally a fourth phase of effluent withdrawal of 10 minutes was applied.

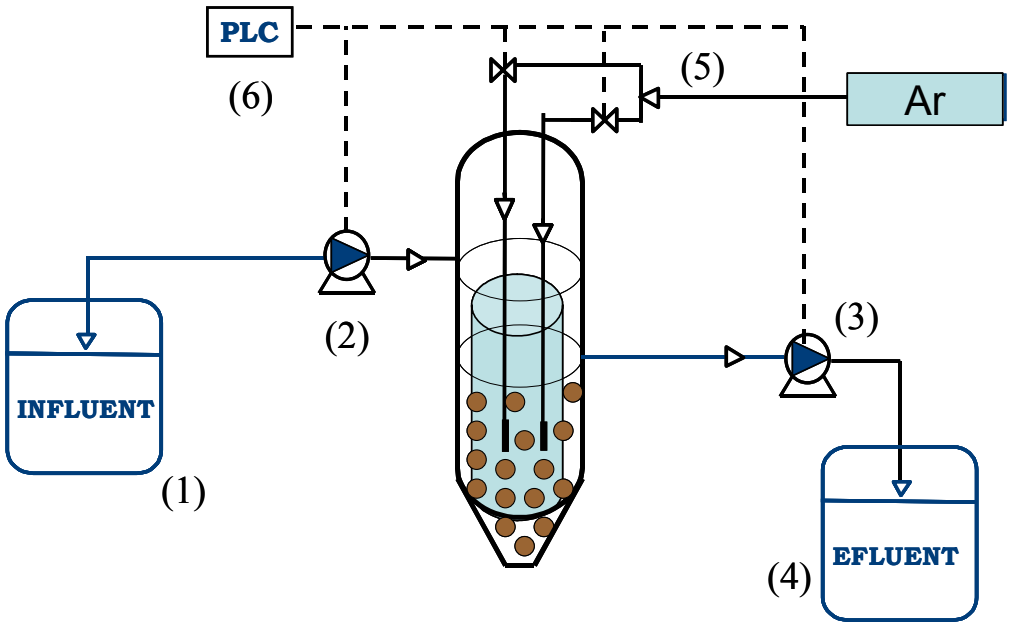


Figure 6.2. Experimental set-up of bubble column and gas lift. (1) Feeding tank; (2) Feeding pump; (3) Effluent pump; (4) Effluent tank (5); Ar valve; (6) PLC.

6.3.2. Feeding media

The reactors were fed with the synthetic media described in Table 6.2. The feeding was appropriately diluted during the first periods of operation until stable conditions were achieved.

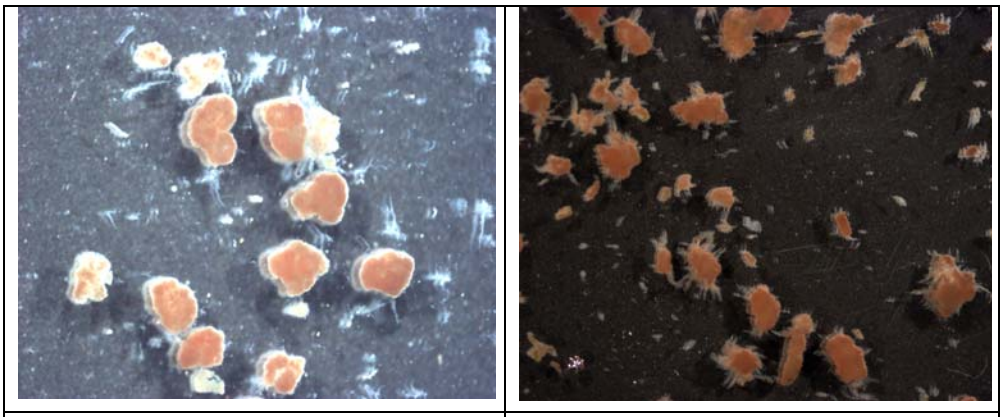
Table 6.2. Composition of the feeding mineral media.

Synthetic wastewater	**g/L**
$NaNO_2$	0.739
$(NH_4)_2SO_4$	0.707
$NaNO_3$	0.425
$KHCO_3$	1.250
NaH_2PO_4	0.050
$CaCl_2 \cdot 2H_2O$	0.300
$MgSO_4 \cdot 7H_2O$	0.200
$FeSO_4$	0.0063
EDTA	0.0063
Trace elements solution*	1.25 mL/L

* Described by van de Graaf *et al.* (1996).

6.3.3. Inoculum

The biomass used as inoculum of the three reactors was, previously to the beginning of this experiment, operated during two years in a SBR at different operational conditions (data not shown) (Dapena-Mora *et al.*, 2004a). This Anammox biomass was already in the form of granules (Figure 6.3A and B) and the initial biomass concentrations added to each reactor at the beginning of the experiment were: SBRM: 1.7 g VSS/L; SBRF1: 0.5 and 2.0 g VSS/L in the first and second inoculation, respectively; and SBRF2: 1.5 g VSS/L.



A) B)

Figure 6.3. A) SBRM Inoculum (10x); B) SBRF1 and 2 Inoculum (10x).

6.3.4. Analytical methods

The pH, nitrate, ammonia, TOC, volatile suspended solids (VSS), total suspended solids (TSS) and SVI were determined according to Standard Methods (APHA, 1999) as described in Chapter 2.

Biomass density, in terms of biomass per volume occupied by the granules (g VSS/(Lgranules)), was determined using dextran blue, which is not absorbed by the biomass (Jiménez *et al*., 1988) and following the methodology proposed by Beun (Beun *et al*., 1999). The dimensions of the granules were measured regularly by using an Image Analysis system. Images of the granules were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (Stemi 200-C, Zeiss). For the digital image analysis the programme Image ProPlus was used. Specifically the programme served to calculate the average feret diameter of the granules. The feret diameter was calculated as the average value between the longest and the shortest segment measured in the granule (Section 2.3 of chapter 2).

Morphological studies of the biomass were performed with a scan electron microscope (Digital SEM Leica 440 at 20 kV) controlled with a computer system and with a magnification capacity ranging from 15 to 290000 folds. The sludge sample was washed with phosphate buffer and subsequently fixed with a solution

of glutaraldehyde and then the samples were dehydrated using ethanol (Section 2.3 of chapter 2).

Fluorescence in Situ Hybridization technique

Anammox population was followed by the Fluorescence in Situ Hybridization technique (Chapter 2, section 2.4). Samples from the reactor were collected (on day 5) and fixed according to Amann *et al.* (1995) with 4% paraformaldehyde as described elsewhere. Hybridization was performed at 46 ºC for 90 minutes adjusting formamide concentration at the percentages shown in Table 6.3. The used probes for in situ hybridization were 5' labelled with the dyes FLUOS or Cy3. Fluorescence signals of disaggregated samples were recorded with an Axioskop 2 epifluorescence microscope (Zeiss, Germany).

Table 6.3. Oligonucleotide probes

Probe	Probe sequence (5'→3')	% FA	Target organisms	Ref.
EUB338I	GCT GCC TCC CGT AGG AGT	20	Bacteria domain	[1]
EUB338II	GCA GCC ACC CGT AGG TGT	60	Bacterial lineages not covered by probe EUB338. Planctomycetales	[2]
EUB338III	GCT GCC ACC CGT AGG TGT	60	Bacterial lineages not covered by probe EUB338 and EUB338II. Verrucomicrobiales	[2]
PLA46	GAC TTG CAT GCC TAA TCC	30	Planctomycetales	[3]
Amx820	AAA ACC CCT CTA CTT AGT GCC C	40	Anaerobic ammonium-oxidizing bacteria *Candidatus* "Brocardia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis"	[4]
Amx368	CCT TTC GGG CAT TGC GAA	15	*All Anammox bacteria*	[5]

[1] Aman *et al.*, 1990; [2] Daims *et al.,* 1999); [3] Neef *et al.*,1998; [4] Schmid *et al.*, 2001; [5] Schmid *et al.*, 2003.

6.3.5. Specific Anammox activity assays

The batch assays used to estimate the Anammox activity were performed according to the methodology described by Buys *et al.* (2000) and slightly modified by Dapena-Mora *et al.* (2006). Completely closed vials with a total volume of 38 mL with 25 mL of liquid volume were used to perform the Anammox batch assays. Biomass concentration at the beginning of the

experiment was fixed around 1.5 g VSS/L. Before the beginning of the batch test the biomass was washed three times with phosphate buffer (0.143 g KH_2PO_4/L and 0.747 g K_2HPO_4/L). The pH value was fixed at 7.8 and temperature at 30 ºC. Gas and liquid phases were purged with argon gas to remove O_2. Initial concentrations of substrates were 70 mg NH_4^+-N/L and 70 mg NO_2^--N/L. The production of N_2 was determined in the gas phase as the increment of pressure in the headspace of the vials, measured by means of a pressure transducer device. Maximum Specific Anammox Activity (SAA) was estimated from the maximum slope of the curve described by the cumulative N_2 production along the time and related to the biomass concentration in the vials.

6.3.6. Operational conditions

The Hydraulic Retention Time (HRT) in the three reactors was fixed at 1 day and the applied Nitrogen Loading Rate (NLR) was maintained between 0.05-0.3 g N/(L·d) by maintaining inlet ammonium and nitrite concentrations between 25-150 mg NH_4^+-N/L and 25-150 mg NO_2^--N/L, respectively. Nitrite was the limiting substrate because of its toxic effect on the Anammox biomass.

The operational strategy consisted of increasing the mechanical stress applied to the reactor SBRM by stepwise variation of the stirring speed between 60 and 250 rpm and increasing stress forces applied to the reactors SBRF1 and 2 by stepwise variation of the gas upflow velocity between 3.52 and 12.35 cm/min (Table 6.4). During the whole operational period the biomass in the effluent was settled and returned to the reactors in order to avoid biomass washout.

Table 6.4. Variation of the flow and upflow velocity.

Reactor	Flow (L/min)	Fluidization velocity (L/(L·min))	Upflow velocity (cm/min)
SBRF1	0.70	0.47	12.35
SBRF2	0.20	0.13	3.52
	0.30	0.20	5.29
	0.42	0.28	7.39
	0.55	0.37	9.70

6.3.7. Calculations

Specific power

SBRM

The power consumption in a stirred vessel depends on the various geometrical parameters of the impeller (diameter (d) and height (h)) and reactor diameter (D) and height (H), the rotating speed (N) and the fluid properties (density (ρ) and viscosity (μ)). From dimensional analysis this relationship can be put into a compact form as the Newton number (Ne) as a function of different parameters (equation [6.2]) (Henzler, 2000). The Ne number is defined as an adimensional number which expresses the ratio between pressure energy and kinetic energy.

$$Ne = f(Re, d/D, H/D, impeller) \quad [6.2]$$

The obtained expression [6.3] for the Ne module is:

$$Ne = \frac{P}{\rho \cdot N^3 \cdot d^5} \quad [6.3]$$

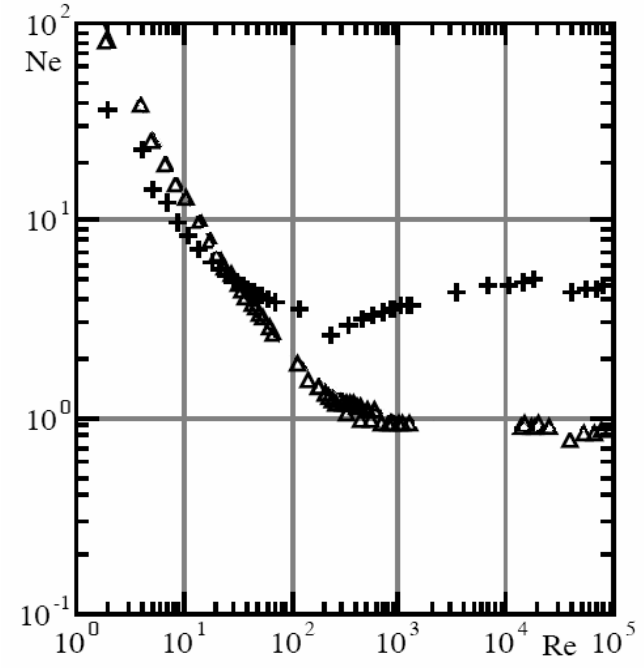
The Reynolds number (Re) referred to the stirrer can be calculated by means of expression [6.4]:

$$\mathrm{Re} = \frac{\rho \cdot N \cdot d^2}{\mu} \quad [6.4]$$

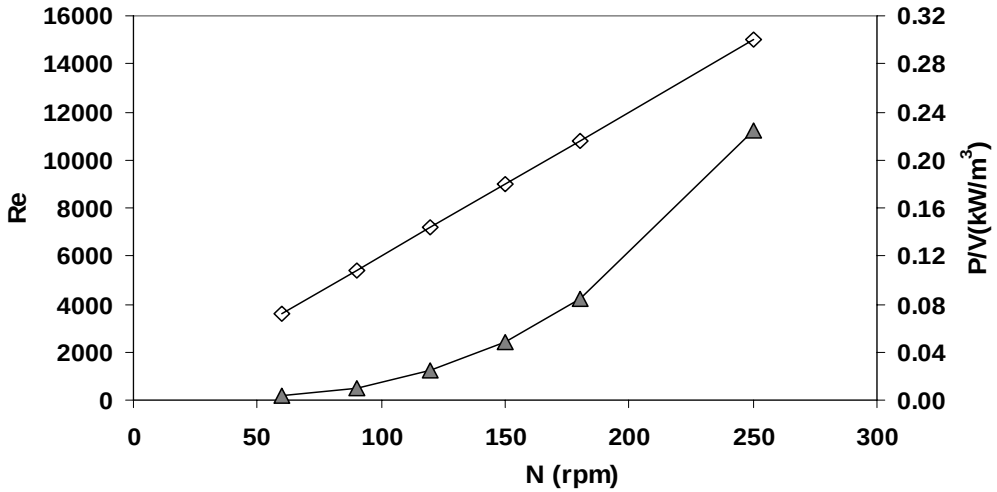
For the case of a standard Rushton turbine the Newton number (Ne) can be estimated from Figure 6.4, which represents the Ne as a function of the Reynolds Number. The input power (P) can be calculated as a function of the stirring speed from equation [6.3] according to the equation [6.5].

$$P = Ne \cdot \rho \cdot N^3 \cdot d^5 \quad [6.5]$$

The value of the Reynolds number varied from 4000 to 15000 during the operational period. As Ne number has a constant value of 4 for Re values higher than 1000 (Figure 6.4A), the input power only depended on the stirring speed (N) (Figure 6.4B). The specific power applied ranged then between 0.003 and 0.23 kW/m^3.



A)



B)

Figure 6.4. A) Newton number versus Reynolds number for the hyperboloid (Δ) and Rushton (+) impellers (Pinho *et al*., 1997). B) Reynolds number (Re) (◇) and specific power (P/V) (▲) as a function of the stirring speed (rpm).

SBRF1 y SBRF2

The power consumption in a gas flow reactor, which depends on the flow rate (F), can be easily calculated by the equation [6.6] (Bang *et al.*, 1998):

$$P = \rho \cdot g \cdot F \qquad [6.6]$$

The value of the Reynolds number varied from 4000 to 17000 during the operational period. The specific power applied ranged then between 0.02 and 0.10 kW/m^3 (Figure 6.5).

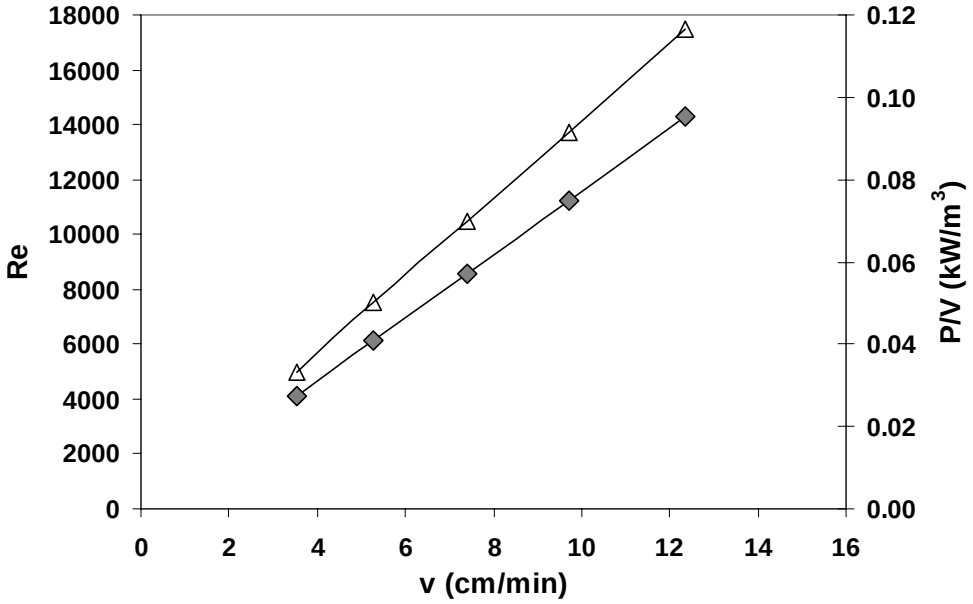


Figure 6.5. Reynolds (Δ) and specific input power (■) versus upflow velocity.

6.4. Results and discussion

The results of the operation characteristics and nitrogen removal efficiencies of each reactor (SBRM, SBRF1 and SBRF2) are firstly discussed, followed by the corresponding biomass characterisation based on its settling properties, sizes distribution of the granules, activity measurements and image analysis observations.

6.4.1. Nitrogen removal efficiency

The reactor SBRM was operated during 218 days (Figure 6.6A). Initially, in the period of time comprehended between days 40 and 50 of operation, an accumulation of nitrite took place as a consequence of an accidental shock of pH in the reactor. Once the reactor efficiency was restored the stirring speed was stepwise increased from 90 to 180 rpm until day 146. At this point nitrite was almost fully depleted in the system (98%) and the average specific nitrogen removal rate in the reactor was around 0.2 g N/(g VSS·d). From this day on, due to the effect of the increase of the stirring speed to 250 rpm (a specific input power of 0.23 kW/m^3), accumulations of 45 - 60 mg NH_4^+-N/L and 35 - 45 mg NO_2^--N/L were detected. Since nitrite was not completely removed, nitrogen gas was also produced during the settling phase causing biomass floatation. On day 180, the stirring speed was decreased again to 90 rpm and after 10 days the reactor restored its complete efficiency.

The reactor SBRF1 was operated during 140 days at an upflow velocity of 12.35 cm/min (specific input power of 0.1 kW/m^3). The applied NLRs to the reactor reached maximum values of 0.3 g N/(L·d) and were decreased to 0.05 g N/(L·d) by means of reducing the concentrations of nitrogen compounds ($NH_4^+$and NO_2^-) to 25 mg N/L each, as it is shown in Figure 6.6B. Initially, in the period of time comprehended between days of operation 15 and 60, accumulation of 5 - 40 mg NO_2^--N/L took place, which made the process unstable. Since nitrite was not completely removed, the efficiency of the process was completely lost and as a consequence the NLR was reduced. On day 60 the reactor was reinoculated with similar amount of biomass as in the previous experiment. Although during a few days, the reactor efficiency was restored and nitrite was almost fully depleted (98%) with an averaged specific nitrogen removal rate

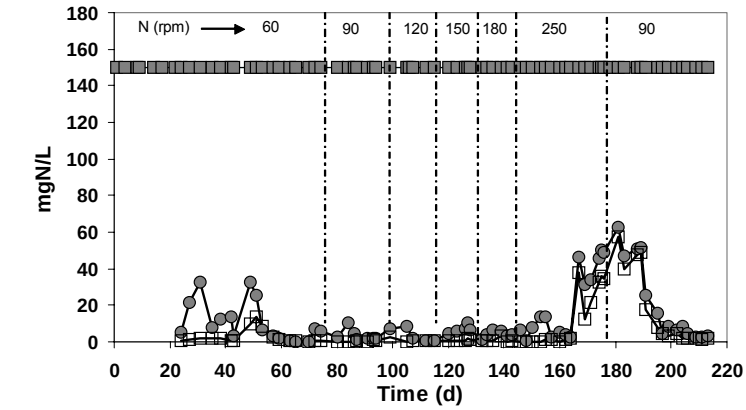
around 0.3 g N/(L·d) the same pattern was repeated. From day 80 on, accumulations of 20-45 mg NH_4^+-N/L and 5-25 mg NO_2^--N/L were detected and the reactor stable operation was not possible.

The reactor SBRF2 was operated during 110 days applying to the reactor different upflow velocities (from 3.53 to 9.70 cm/min) in order to expose the system to different specific input powers, ranging between 0.027 kW/m^3 and 0.075 kW/m^3. During the first two steps, nitrite (limiting substrate) removal percentage was around 98%. However, when the upflow velocity was increased to 7.39 and 9.70 cm/min (an input power of 0.057 and 0.075 kW/m^3), accumulations of 30-100 mg NH_4^+-N/L and 10-75 mg NO_2^--N/L were detected (Figure 6.6C). Since nitrite was not completely removed, nitrogen gas was also produced during the settling phase causing biomass floatation. On day 80, the upflow velocity was decreased again to 3.53 cm/min but not restoration of the nitrogen removal efficiency was achieved after three weeks of operation.

Floatation appeared in the three reactors when stages of nitrite accumulation occurred. This phenomenon was previously observed by Dapena-Mora *et al.* (2004b) and Arrojo *et al.* (2006).

In reactor SBRM for the values of the specific input power of 0.003 - 0.09 kW/m^3 (corresponding to 60 - 180 rpm) the value of activity was practically constant around 0.40 g N/(g VSS·d) (Figure 6.7A) . However, this value decreased to 0.25 g N/(g VSS·d) when the specific input power applied to the reactor was 0.23 kW/m^3 (250 rpm) but the nitrogen loading rate (NLR) was maintained constant 0.3 gN/L·d.

In the case of SBRF1 and 2 the values of the SAA were practically constant around 0.35 g N/(g VSS·d) (Figure 6.7B; 6.7C) for the specific input power comprehended between 0.027 kW/m^3 and 0.041 kW/m^3. However, this value decreased to 0.15 g N/(g VSS·d) when the specific power applied to the reactor was 0.057 kW/m^3. The activity decreased to 0.05 g N/(g VSS·d) when the specific input power was increased to 0.075-0.095 kW/m^3. For this reason, the nitrogen loading rate applied to the reactor SBRF1 decreased under 0.05 gN/L·d.



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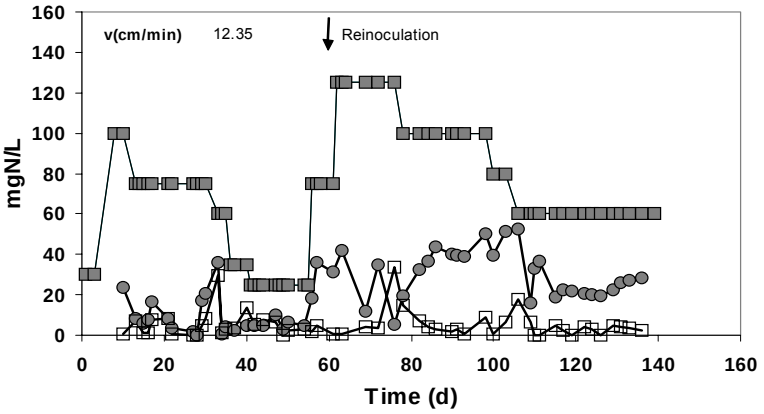

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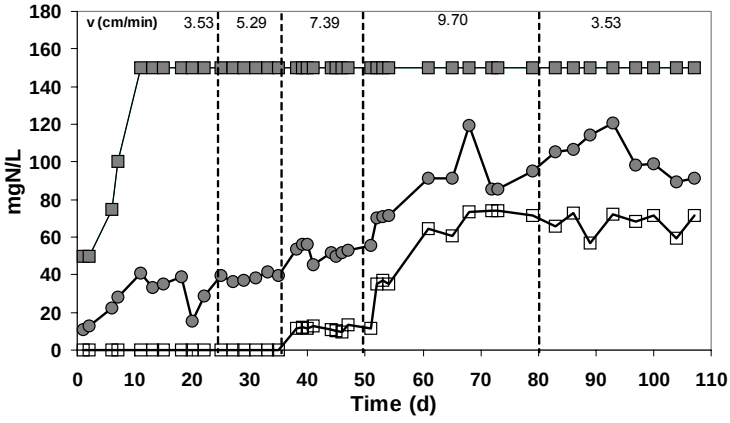
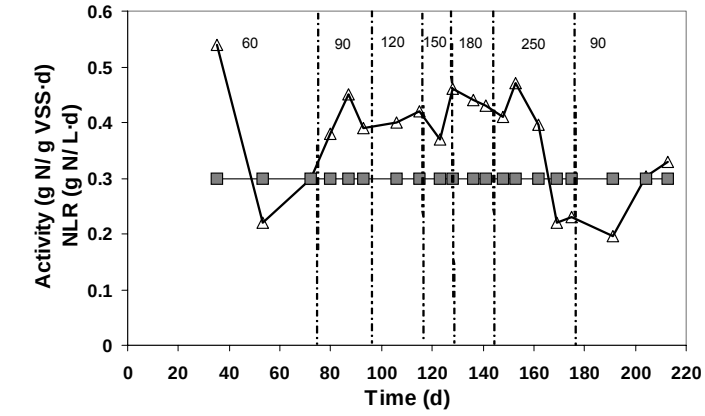


C)

Figure 6.6. Nitrogen compounds in the SBR: influent NH_4^+, NO_2^- (■), effluent NH_4^+ (●), NO_2^- (□). A) SBRM, B) SBRF1 and C) SBRF2.



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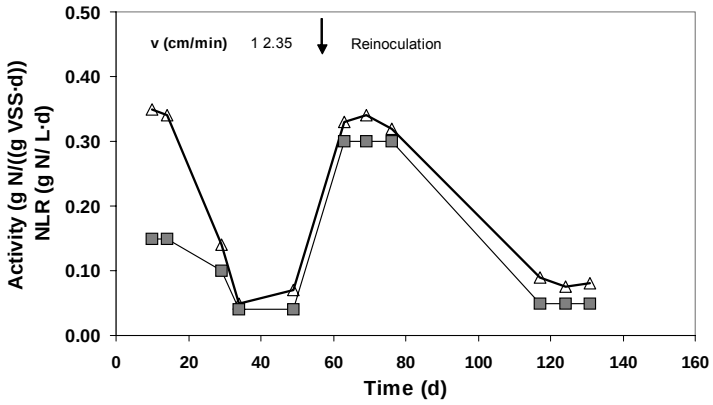

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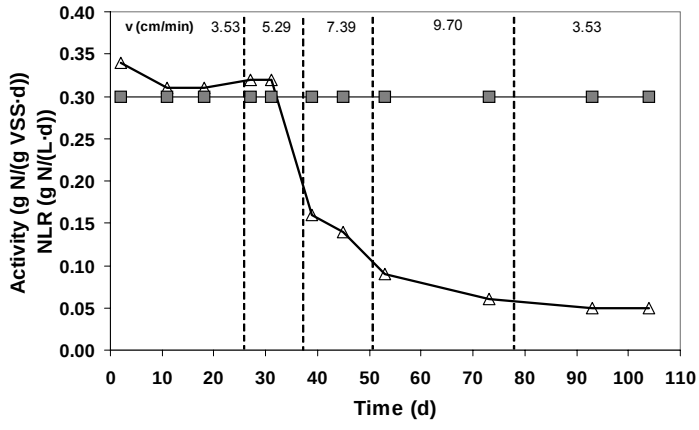


C)

Figure 6.7 Specific activity (Δ) and NLR (■) applied to the reactors. A) SBRM, B) SBRF1 and C) SBRF2.

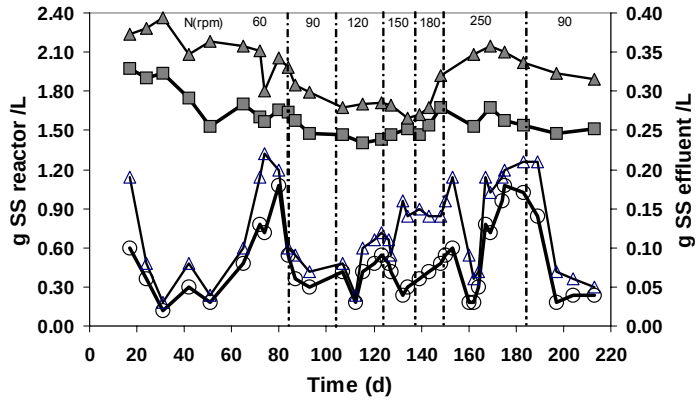
6.4.2. Biomass evolution

Solids concentration

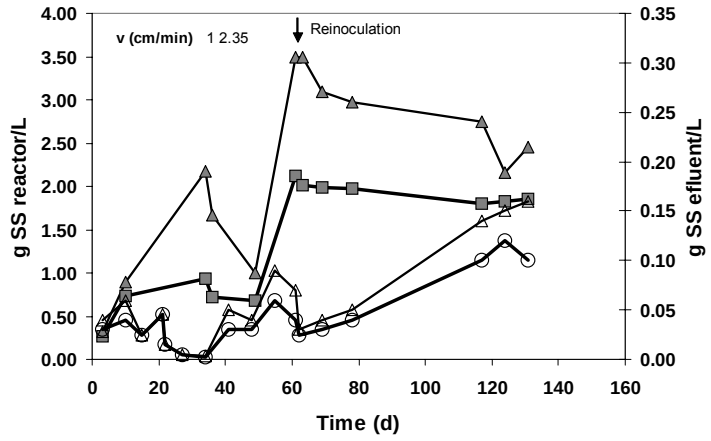
Biomass concentration in the reactor SBRM was 1.6-1.8 g VSS/L at the beginning of the experiment (Figure 6.8A) and the ratio of VSS/TSS ranged from 75% to 90% during the whole operation. The TSS concentration in the effluent was comprehended during most of the experimental period between 0.04 and 0.09 g TSS/L and the VSS were between 0.03 and 0.07 g VSS/L. However, at the beginning of the experiment and as a consequence of a shock of pH, the solids concentration in the effluent increased up to 0.20 g TSS/L and to 0.15 g VSS/L. After the operation day 146, when the stirring speed was fixed at 250 rpm the concentration of solids also increased up to 0.20 g TSS/L presumably as a result of the increase of the shear stress applied to the system. However, on day 180 when the rotating speed was turned to 90 rpm the concentration of solids in the effluent decreased.

In the case of SBRF1 the biomass concentration was initially of 0.7 and 3 g TSS/L and or 0.5 and 2.0 g VSS/L for the first and the second inoculation of the reactor. The TSS concentration in the effluent was comprehended during most of the experimental period between 0.04 and 0.09 g TSS/L and the VSS were between 0.03 and 0.07 g VSS/L. However, at the end of the experiment as a consequence of the instability of the process, the solids concentration in the effluent increased up to 0.20 g TSS/L and to 0.15 g VSS/L (Figure 6.8B). The instability of the process indicated that the shear stress which was exposed the system (a specific input of 0.1 kW/m^3, according to Figure 6.4A) was so high that a success operation was not allowed. So, a reduction of the upflow velocity was proposed by means of the use of a new configuration of the reactor. The new configuration consisted on the insertion of a riser in the reactor to obtain a gas-lift SBRF2. In this way the needed upflow velocity to obtain complete mixture in the system was reduced and a wider range of velocities was studied.

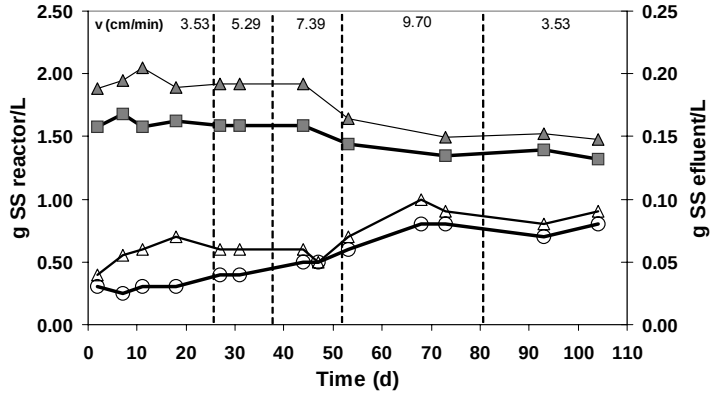
The concentration of the biomass in the SBRF2 was maintained practically constant around 1.5 gVSS/L. The TSS concentration in the effluent trended to increase during the last periods of operation (upflow velocities up 7.39 cm/min), achieving a stable value around 0.1 g TSS/L and 0.08 g VSS/L (Figure 6.8C).



A)



B)



C)

Figure 6.8. Concentrations of TSS (▲), VSS (■) in the reactor and TSS (Δ), VSS (0) in the effluent of the reactor. A) SBRM, B) SBRF1 and C) SBRF2.

This increment of biomass concentration in the effluent of all the reactors might have been originated either by the breaking up of the granules into smaller fragments with lower settling abilities or by direct floatation of the biomass due to nitrite accumulation, both phenomena leading to biomass washout.

Biomass density and sludge volumetric index (SVI)

The biomass density of SBRM was in the range from 25 to 40 g VSS/($L_{granules}$) during most of the experimental period. However, at the end of the experimental operation the density was slightly increased to values in the range of 40-55 g VSS/($L_{granules}$) (Figure 6.9A)

The density of the granules in SBRF1 was between 30 and 50 g VSS/($L_{granules}$) during most of the experimental period . However, at the end of the experimental operation the density was slightly increased to values in the range of 50-65 g VSS/($L_{granules}$).

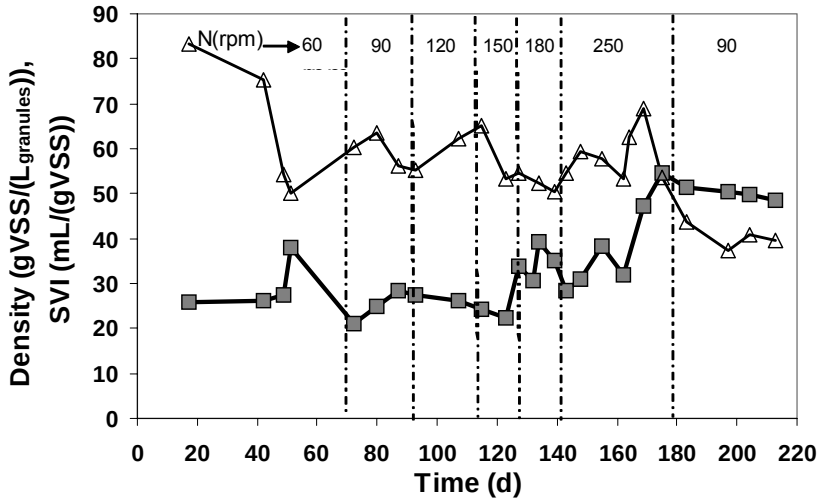
The density of the granules from SBRF2 was between 30 and 60 g VSS/($L_{granules}$) during most of the experimental period (Figure 6.9B) corresponding the highest values to those periods with the highest gas upflow velocities.

The biomass density obtained during most of the operation of the three reactors was similar to the value reported for aerobic granules or biofilms formed in airlift reactors of 25-60 g VSS/($L_{granules}$) (Kwok *et al.*, 1998) and higher than those reported by Beun *et al.* (1999) of 11.9 g VSS/($L_{granules}$), 10-15 g VSS/($L_{granules}$) (Arrojo *et al.*, 2004; Mosquera-Corral *et al.*, 2005) and 15-20 g VSS/($L_{granules}$) (Tijhuis *et al.*, 1994). Shear stress was found to strongly influence the biomass density in the way that the higher the applied stress the greater the density of the biofilms (Kwok *et al.*, 1998). In the case of formation of aerobic granules the shear stress was found to probably stimulate the bacterial secretion of extracellular polysaccharides, which might contribute to the compactness and strength of the granular structure (Ohasshi and Harada, 1994; Liu and Tay, 2004).

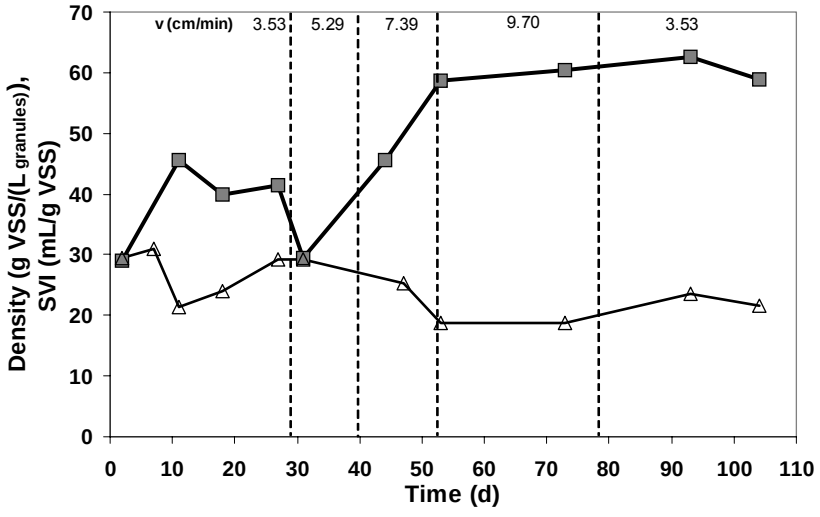
The Sludge Volumetric Index (SVI) of the sludge in SBRM was around 55 - 60 mL/(g VSS) during most of the steady-state period (Figure 6.9A).

Nevertheless, at the end of the experiment it decreased slightly to 40 mL/(g VSS). The SVI was not measured from the sludge of SBRF1.

The Sludge Volumetric Index (SVI) in SBRF2 was around 25 - 30 mL/(g VSS) during most of the steady-state period. However, at the end of the experiment trended to decrease slightly to 20 mL/(g VSS).



A)



B)

Figure 6.9. Evolution of biomass density (■) and SVI (Δ) in the SBR. A) SBRM, B) SBRF2.

The obtained results indicate that the Anammox granules became more compact at higher stress. The compactness can be related to the granules break up due to the increase of hydrodynamic stress, in such a way that the smaller granules settle forming a more compact bed. The weaker attached biomass on the surface of the aggregates was easily removed. Similar results were obtained in aerobic biofilm and granular systems where stress was caused by means of different aeration flows in airlift and column reactors, respectively (Kwok *et al*., 1998; Tay *et al.*, 2004). These authors considered that high upflow air velocity could help to remove the fluffy and less dense sludge, thereby an increase biomass density of granules result in a decrease in the SVI.

This removal of the weak parts in the surface of the granules can be corroborated with the gradual decrease of the granules average size along the operational time (Figure 6.10). These results clearly show that the breakage of the granules was a process that occurred when the shear stress was increased.

Granules size distribution

The size distributions of the granules in SBRM were measured at different stirring speeds conditions (60, 90, 120, 150, 180 and 250 rpm). Most of the volume percentage of the biomass at stirring speed comprehended between 60 and 180 rpm corresponded to granules with a size distribution between 0.65 and 3.00 mm (Figure 6.10) with an average feret diameter of 0.64 mm (Figure 6.11). Great difference in the diameters distribution between the experiments performed at different rotational speeds was not found except for the last value tested (250 rpm). However, at 250 rpm (on days 169 and 175) the percentage of the biomass in terms of volume was shifted to smaller diameters indicating the contribution of the small granules presented in the system. The average feret diameter in this period was of 0.35 mm. The average feret diameter of the granules was decreased in 45 % referred to the value at 90 rpm.

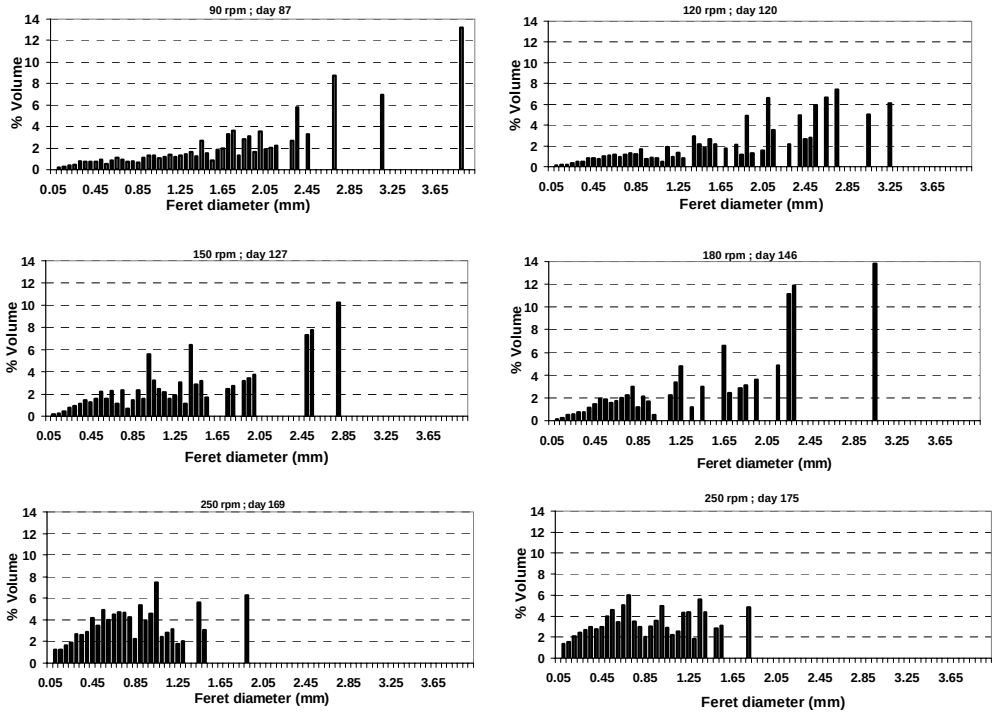


Figure 6.10. Comparison of the size distribution between granules on different operating days in SBRM.

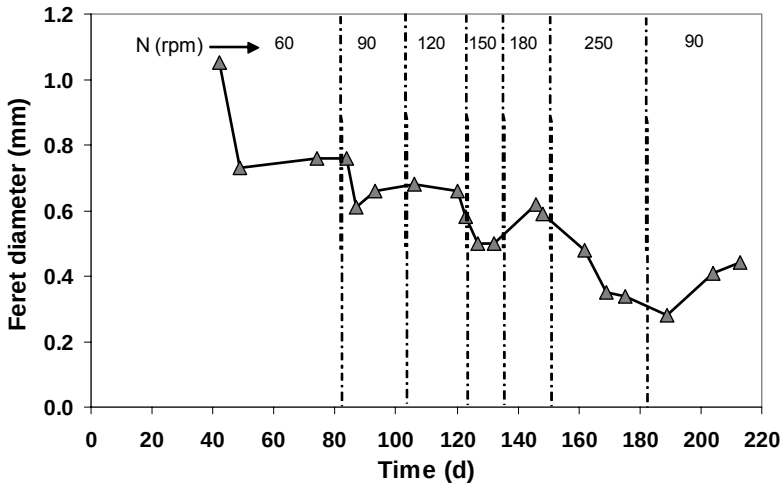


Figure 6.11. Distribution of average feret diameters along of the operation time in SBRM.

Concerning to the size distributions in SBRF1, the average feret diameter indicated a reduction of the size of the granules along the operation time. At the beginning of the experiment, the average feret diameter was around 0.7 mm. However, on day 100 this value decreased to 0.35 mm, which indicated a 50% of reduction (Figure 6.12; 6.13).

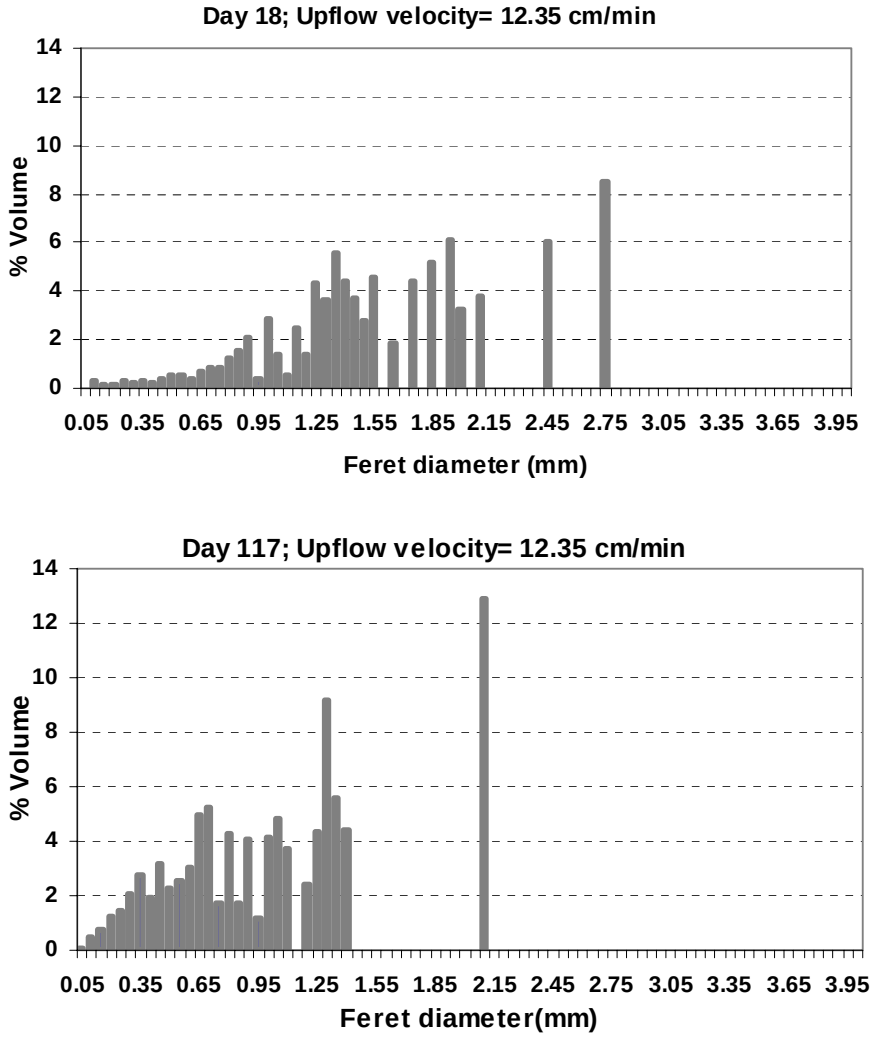


Figure 6.12. Comparison of the size distribution between granules on different operating days in SBRF1.

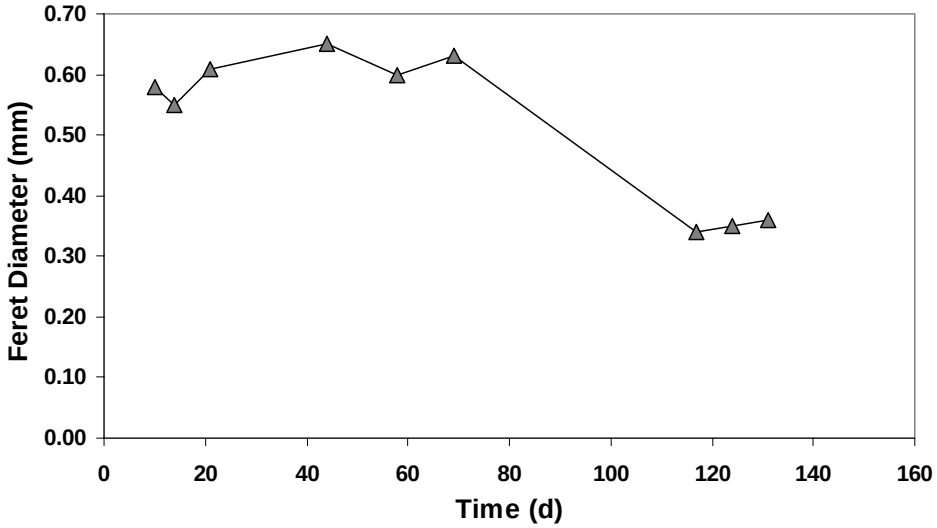


Figure 6.13. Average feret diameter along the operation time in SBRF1.

In the case of SBRF2 the size distributions indicated an evolution at different upflow velocities (3.53, 5.29, 7.39, 9.70 cm/min).

At the beginning the average diameter of the granules was comprehended between 0.75-0.85 mm (Figure 6.14 and 6.15). Significant difference in the diameters distribution between the experiments performed at different upflow velocities was not found except for the last value tested (9.70 cm/min). The average feret diameter in this period was 0.55-0.6 mm. The average feret diameter of the granules decreased in 30% referred to the value at 3.53 cm/min. During this period biomass aspect became fluffy, irregular and with loose morphology (Figure 6.18B).

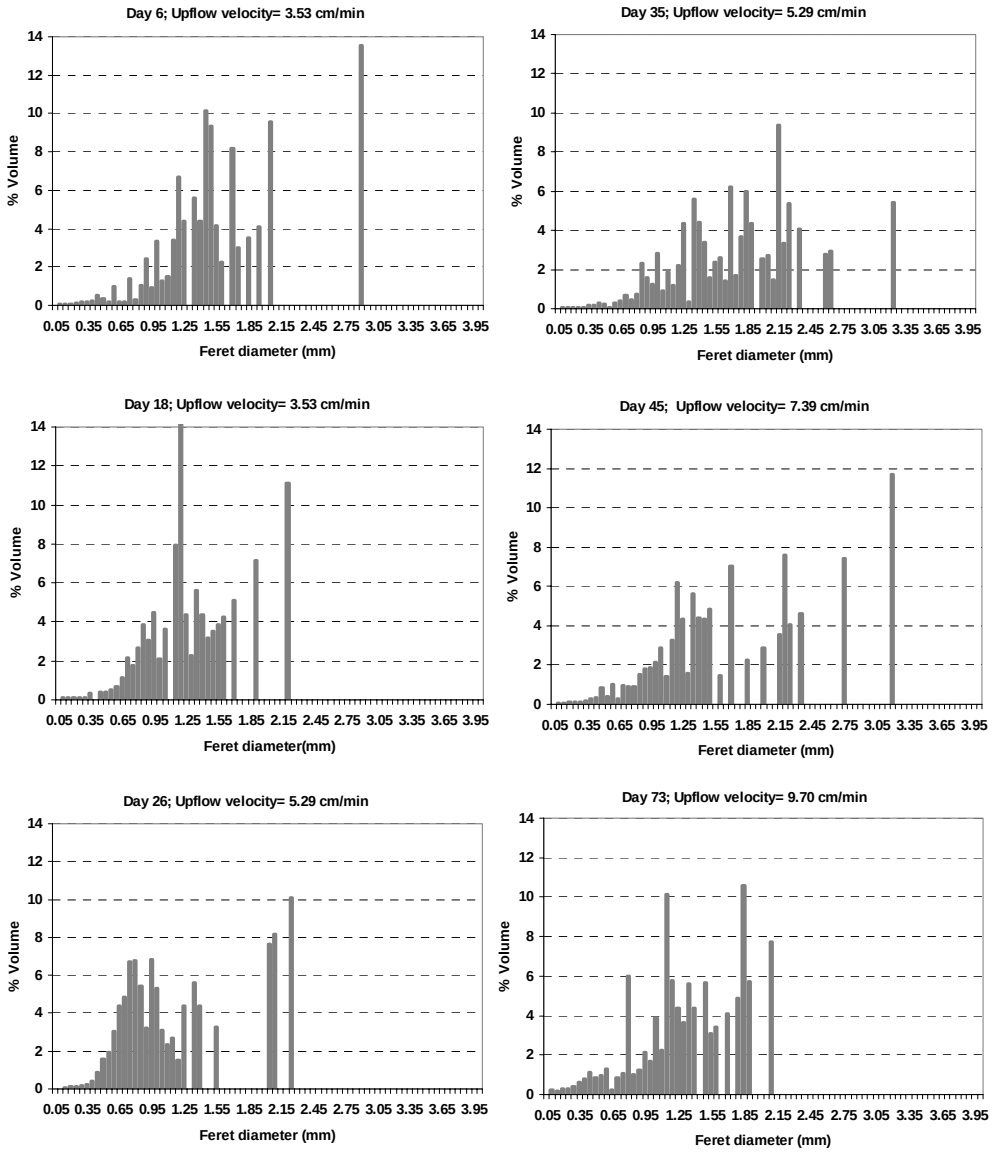


Figure 6.14. Distribution of average feret diameters along of the operation time in SBRF2.

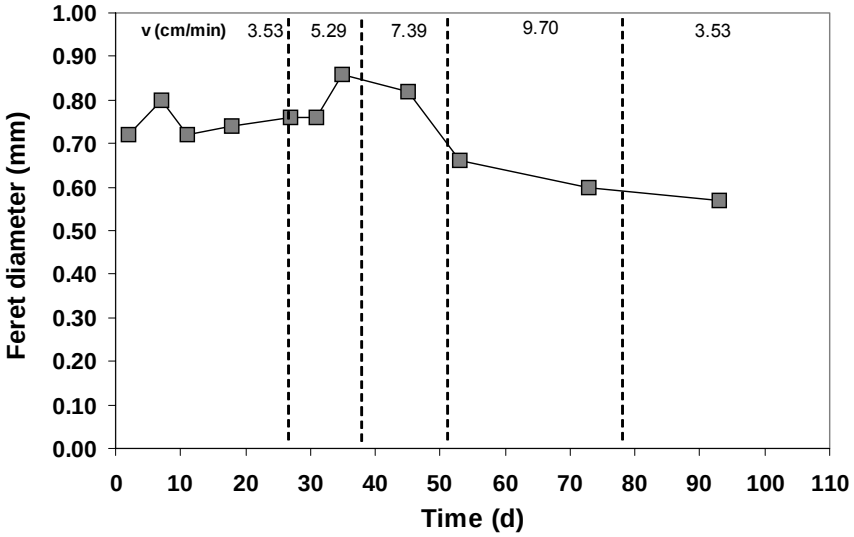
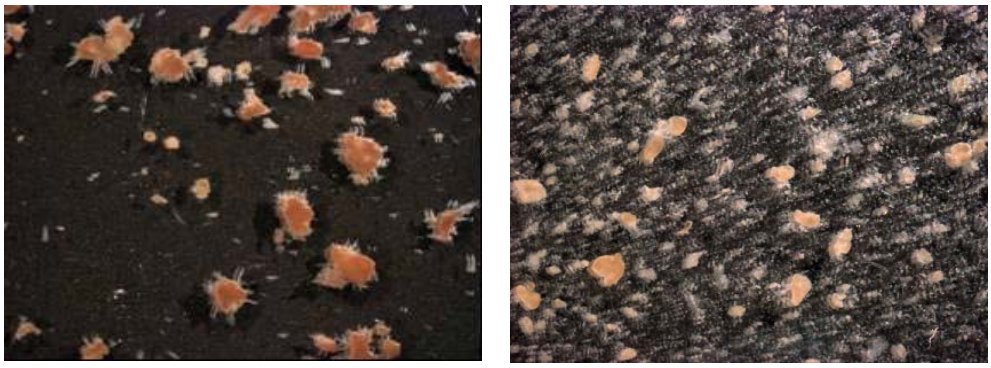


Figure 6.15. Distribution of average feret diameters along of the operation time in SBRF2.

Granules appearance

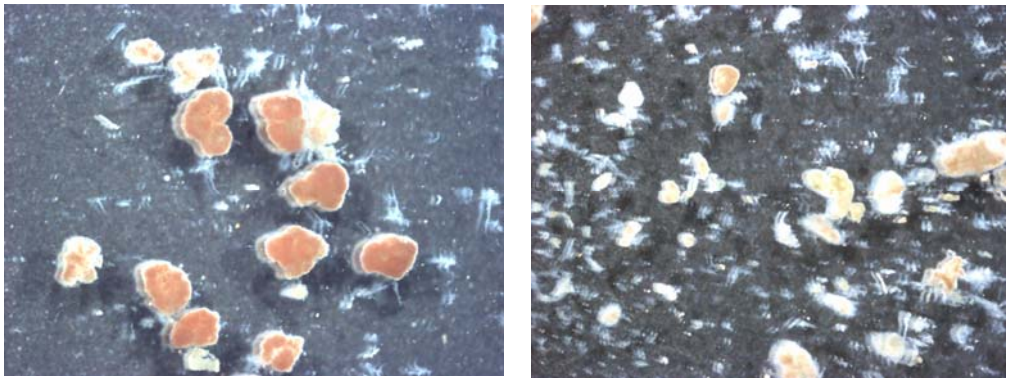
The sludge used in the system as inoculum was a granular sludge and the shape of the granules was round and with a very clear outline (Figure 6.16A, 6.17A, 6.18A). Nevertheless, after a few weeks the formation of small aggregates with an average feret diameter around 0.40 mm was observed (Figure 6.16B, 6.17B, 6.18B). During this period biomass had a fluffy, irregular and loose morphology.

During operation of the reactors, a change of colour, from reddish (typical colour of Anammox biomass, Dapena-Mora *et al*., 2004c) to brownish was observed. This change of colour took place when a high stress was applied to the system and the instability of the process occurred**.**



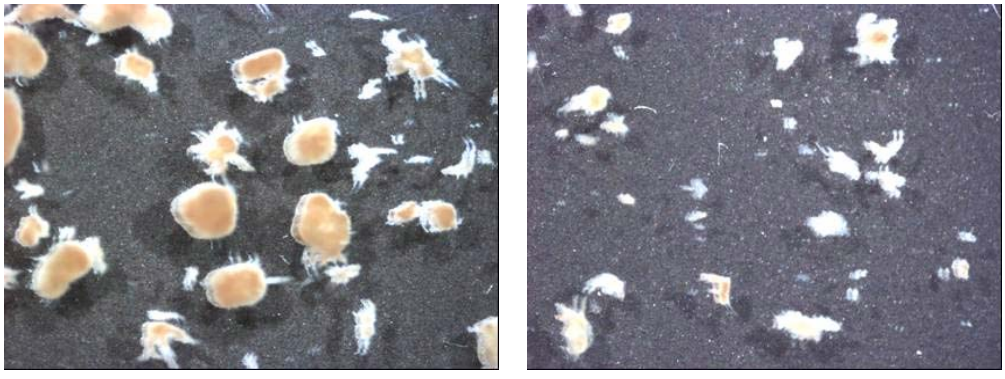
A) B)

Figure 6.16. Stereomicroscope images of the granules (magnification 10x) on days 87 (A) and 175 (B). SBRM



A) B)

Figure 6.17. Stereomicroscope images of the granules (magnification 10x) on days 28 (A) and 117 (B). SBRF1



A) B)

Figure 6.18. Stereomicroscope images of the granules (magnification 10x) on days 6 (A) and 70 (B). SBRF2

Scanning electron microscopy (SEM) was used to visualize the surface of aggregates from SBRM along the experiment (Figure 6.19). The figure represents a sample of a mature granule with a cauliflower like aspect from day 134 and shows a granule structure clearly distinguished.

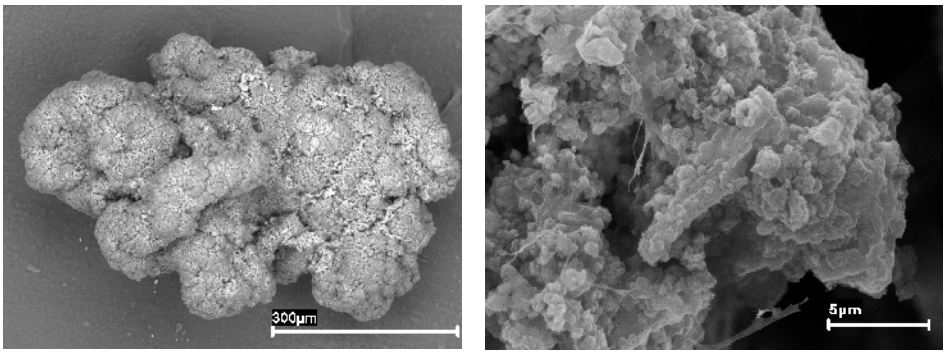


Figure 6.19. Scanning Electron Microscop images of the Anammox granules (day 134) in SBRM.

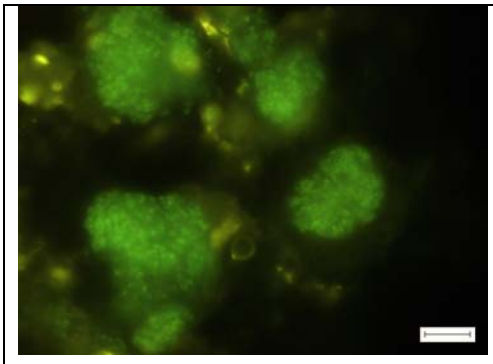
Biomass characterisation

Fluorescence In situ hybridization (FISH) of disaggregated samples revealed that bacteria belonging to the order *Planctomycetales* were the dominant population; positive results were obtained when probes Amx820 or Amx368 were applied. It was no possible to specify which Anammox species were present in the biomass, but they were supposed to be *Candidatus* "Kuenenia Stuttgartiensis"(Dapena-Mora *et al.,* 2004c).

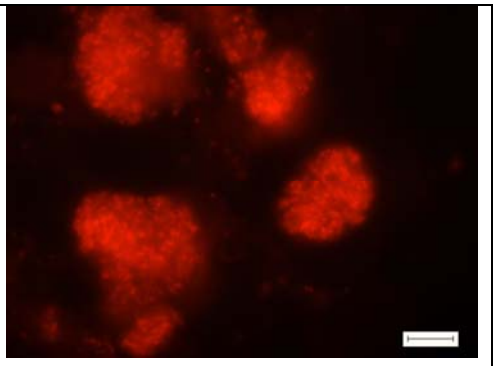
It was observed that majority of the Anammox bacteria formed spherical microcolonies consisting of rod-shaped cells, where to distinguish the internal compartment named as anammoxosome was possible (Figure 6.20; 6.21). Moreover, these microcolonies grown in contact with a high number of precipitates which difficult the observation under microscope (Figure 6.22).

The FISH technique only shows positive hybridization of unbroken cells, it would be interesting to observe the structure after exposing the Anammox to stress conditions, to check if biomass was inactive. According to Schmid *et al.* (2005) in the case of slow growing microorganisms as Anammox a positive signal

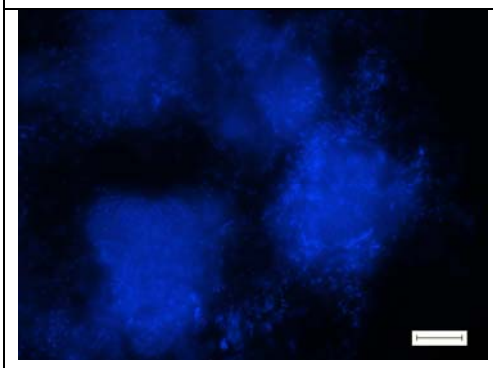
with the FISH technique can be obtained even in the case of starving or inhibited cells.



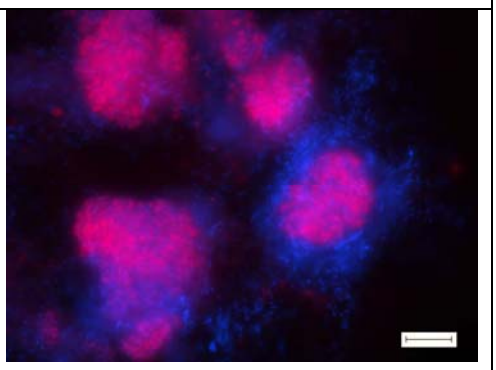
A) Amx820



B) Pla46

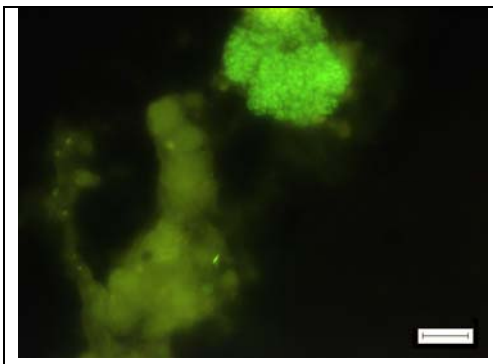


C) DAPI

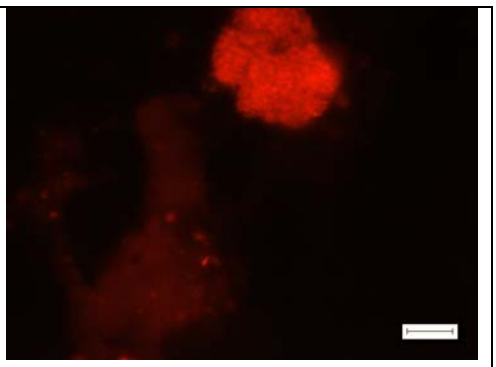


D) Overlaying

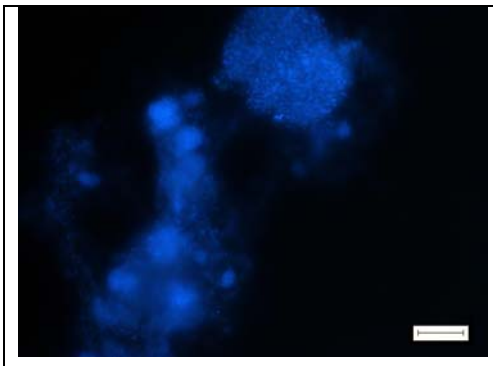
Figure 6.20. FISH analysis on day 5 (bar represent 10 μm) in SBRM.



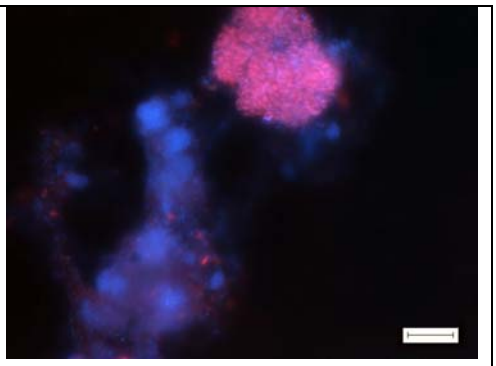
A) Amx368



B) Pla46



C) DAPI



D) Overlaying

Figure 6.21. FISH analysis on day 5 in SBRF2.

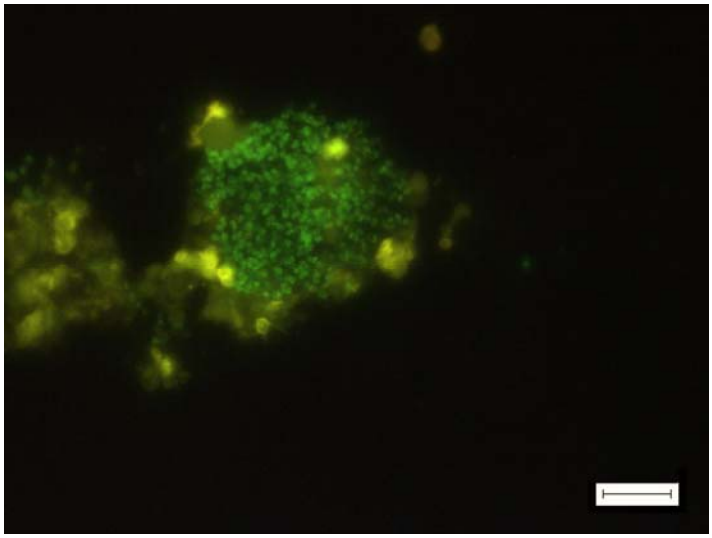


Figure 6.22. FISH analysis on day 5 (precipitates in yellow) in SBRM.

6.4.3. Anammox activity: comparison between hydrodynamic and mechanical stress

A comparison between the effects of mechanical and gas flow stress on Anammox granules in SBRs was studied. The evolution of the specific Anammox activity was measured and related to the specific input power (P/V) applied to the system along the operational period (Figure 6.23A and B).

In reactor SBRM for the values of the specific input power of 0.003-0.09 kW/m^3 (corresponding to 60-180 rpm) the value of activity was practically constant around 0.40 g N/(g VSS·d). However, this value decreased to 0.25 g N/(g VSS·d) when the specific input power applied to the reactor was 0.23 kW/m^3 (250 rpm). In a similar way Chisti (2000) found constant growth rates for cells grown in serum-free batch cultures using a Rushton turbine with stirring speed up to 270 rpm.

In the case of SBRF1 and 2 the values of the SAA were practically constant around 0.35 g N/(g VSS·d) for the specific input power comprehended between 0.027 kW/m^3 and 0.041 kW/m^3. However, this value decreased to 0.15 g N/(g VSS·d) when the specific power applied to the reactor was 0.057 kW/m^3. The activity decreased to 0.05 g N/(g VSS·d) when the specific input power was increased to 0.075-0.095 kW/m^3. At this value, a rupture of the granules occurred and the biomass retention worsened due to the breakage of the granules and a floatation caused by nitrite accumulation.

These results clearly show that the breakage of the granules and the reduction of the activity were a process that occurred when the shear stress was increased independently of how this stress was applied to the system. Figures 6.23a and b show the values of the granule diameter and the specific activity measured depending on the specific input power. The lineal distribution achieved was similar to that found by Henzler (2000). This author also found a strong influence of the specific input power and the impeller type on the disintegration of flocs, so that the effect of the operating conditions and the reactor type.

The SBR has been proved to be a suitable system for the enrichment of a microbial community with an extremely slow growth rate like that performing Anammox process (Strous *et al*., 1998). Dapena-Mora *et al*. (2004c) already reported the growth of Anammox bacteria in form of granules in SBR systems which permit a homogeneous distribution of substrates, products and biomass, an efficient retention of the biomass and the prevention of the formation of local accumulations of nitrite that could inhibit the Anammox process. However, the influence of the shear forces caused by the mechanical stirring and gas upflow velocities in SBR systems where the Anammox process is performed was never studied.

The increase of the stirrer speed or gas upflow velocity, which involves an increase of the shear stress, was found to provoke changes on the properties of biomass aggregates and on the process efficiency. The possible reasons for these effects are now discussed.

In terms of reactor operation the increase of the shear stress up to 250 rpm in SBRM and up to 7.4 cm/min in SBRF provoked a worsening of biomass retention inside the reactor and a decrease of Anammox efficiency. Both combined phenomena have already been observed in previous studies with SBRs when the specific NLRs applied to the reactors exceeded the corresponding Specific Anammox Activity (SAA) of the biomass, which remained practically constant (Dapena-Mora *et al.*, 2004a). Different effect was observed in the present study where the biomass SAA decreased from 0.40 to 0.25 g N/(g VSS·d) when the shear stress increased due to the increase of stirrer speed till 250 rpm and it decreased from 0.35 to 0.10 g N/(g VSS·d) when the gas upflow velocity increased to 7.4 cm/min. At this point the specific NLRs applied were constant and exceeded the SAA causing the instability of the process. Similar decrease of biomass activity in relation to the shear forces was observed by several authors (Jüsten *et al.*,1998; Biedermann, 1994; Bücher, 1993; Casas-López *et al.*, 2005) in systems applied to grow cells. However, the mechanism responsible for this effect has not been yet clearly established.

Chisti (2000) also observed the loss of the cell growth rate when high speeds and gas upflow velocities were used (more than 270 rpm and 9.7 cm/min, respectively) without cellular lysis being involved in the process and keeping more than 97% of the cells viable. This author proposes that sublethal levels of hydrodynamic forces clearly affected cell growth and reproductive processes without cellular breakage or other physical damage. In the present study the reduction of the observed decrease of the SAA of the biomass might be somehow related to cellular lysis. Total Organic Carbon (TOC) concentrations in the liquid media increased from 8.0 to 13.5 mg TOC/L in case of 180 and 250 rpm, respectively. This increase of TOC accounts for the amount of biomass reduction in the reactor from day 170 to day 180 (100 mg VSS/L). Although this reduction of biomass concentration in the system was not enough to justify the decrease of

SAA of the biomass, but it was probably associated to the breakage of the granules.

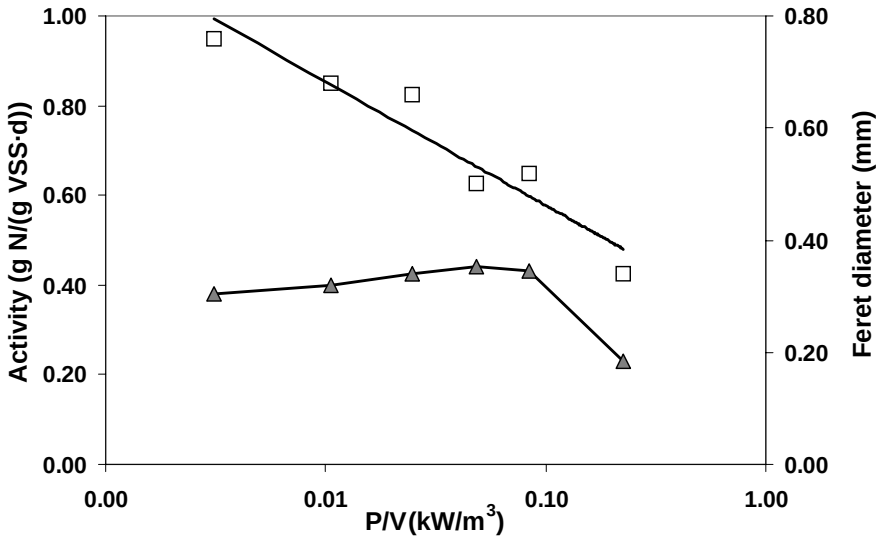
The rupture of the Anammox granules led to smaller size aggregates which should benefit the substrate diffusion (van Benthum *et al.*, 1997) and improve the activity of the biomass placed in the inner cores of the granules with a consequent increment of SAA. Strous *et al.* (1998), working with Anammox granules in a SBR, postulated that 50% of the Anammox biomass was inactive because of substrate limitations inside the granules. Thus, this size reduction should exert a positive effect on the SAA, which was not observed in the reactor.

The rupture of the granules or even the detachment of weak patches of biomass from the surface of the granules involved an increase of suspended solids wash-out of the reactor due to biomass flotation closely related to the nitrite accumulation in the media (Dapena-Mora *et al*., 2004a). However, this trend was not followed by the SVI value, which decreased indicating that the settling properties of the sludge were improved. This effect was related to the fact that the granules were smaller and more compact than in the previous stages. As a consequence the density of the granules experienced an increased due to the rupture of part of the granules.

Another possible reason for the loss of activity could be related to the loss of catabolic and anabolic intermediates to the external environment which can have an important effect on the biomass yield in the case of slow-growing bacteria like Anammox. These bacteria must make up these losses by investing endogenous electron donors (Sinninghe-Damste *et al*., 2002). Hydroxylamine and hydrazine (intermediate compounds of the Anammox process) have been detected experimentally outside the Anammox cells in previous studies. Although in the present study no measurements of these compounds were performed the addition of slight concentrations of hydroxylamine did not favour the Anammox activity.
Besides this phenomenon if relevant could be related to the Anammox cycle, where cytochrome C has an important role. It is currently hypothesized that these c-type cytochromes in “K. stuttgartiensis” is the main responsible for electron transfer from hydrazine to nitrite (Cirpus *et al*., 2005). It could be that the loss of activity was related to the loss of the cytochrome C–hemes, the reduction of

nitrite could not take place and so the typical reddish colour can not be maintained in the Anammox biomass.

A)



B)

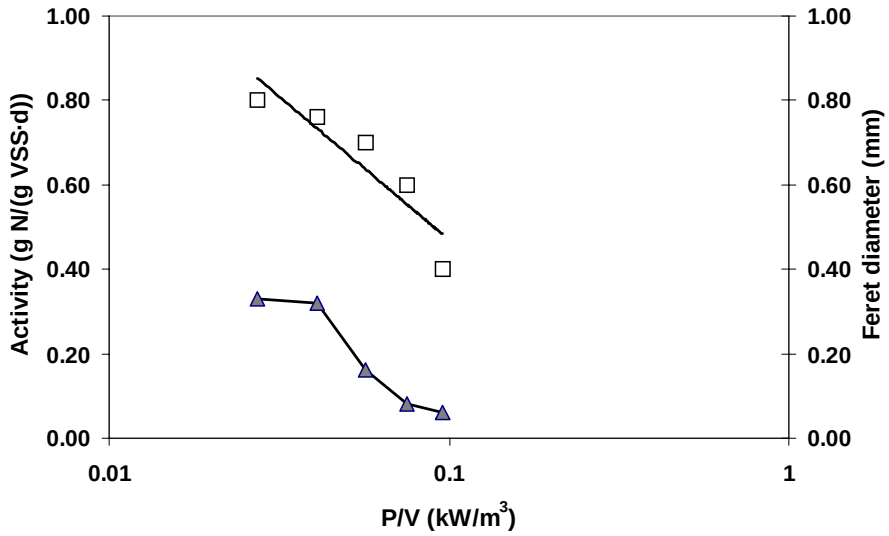


Figure 6.23. Comparison between results of SBRM (A) and SBRF1 and 2 (B). Evolution of the Specific Anammox Activity (▲) and the average feret diameters (□) with the different specific powers tested.

6.5. Conclusions

- The presented results show the high capacity of the Anammox process in order to support the high stress supplied by mechanical stirring or gas upflow velocity in SBR systems. The Anammox process was successfully carried out at specific input power between 0.003 and 0.09 kW/m^3 (up to 180 rpm) for the mechanically stirred reactor and between 0.003 and 0.057 $kW \cdot m^{-3}$ (up to 5.29 cm/min) for the gas upflow recirculating reactor. However, a reduction on the stability of the process took place when the stirring speed was increased to 250 rpm and the gas upflow velocity to 7.4 cm/min, which means a specific input power applied to the system of 0.23 kW/m^3 and 0.075 kW/m^3, respectively.
- This reduction on the stability affected mainly the specific Anammox activity of the sludge and the biomass retention in the system.
- Activity of the Anammox granules was decreased around 50% and 85% for SBRM and SBRF, respectively, which caused a loss of the system efficiency due to a combination of cellular lysis and granules breakage.
- The biomass retention worsened due to the breakage of the granules and floatation caused by nitrite accumulation. However, the granules were more compact and the density was increased.

When operated at full-scale the control of the shear stress in SBR systems is required in order to perform the process in stable conditions. Shear forces affect to the activity of the Anammox system only in case of application of high stress. When the aim is the formation of granular biomass further research must be focused on the determination of the minimum needed shear stress. It is important to highlight that the activity, the size of the granules and the stability of the process was recovered when the initial shear stress were restored.

6.6. References

Alphenaar P.A., Visser A. and Lettinga G. (1993). The effect of liquid upflow velocity and hydraulic retention time on granulation in UASB reactors treating wastewater with a high-sulphate content. *Bioresource Technology,* **43**, 249-58.

Amann R.I. (1995). In situ identification of micro-organisms by whole-cell hybridization with RNA-targeted nucleic acid probles, p.1-15. In A.D.L.: Akkerman, J.D., van Elsas and F.J. de Brujin (ed), molecular microbial ecology manual. Kluwer Academic Publisher, Dordrecht, The Netherlands.

APHA-AWWA-WPCF. (1999). Standard Methods for the examination of water and wastewater. 20th Edition. Clesceri, L.S., Greenberg, A.E. & Eaton, A.D. (eds.).

Arrojo B., Mosquera-Corral A., Garrido J.M. and Méndez R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research,* **38**, 3389-3399.

Arrojo B., Mosquera-Corral A., Campos J.L. and Méndez R. (2006). Effects of mechanical stress on Anammox granules in a sequencing batch reactor (SBR). *Journal of Biotechnology*, **123**, 453-463.

Arcand Y., Guitot SR., Desrochers M. and Chavarie C. (1994). Impact of the reactor hydrodynamics and organic loading on the size and activity of anaerobic granules. *Chemical Engineering Journal,* **56**, 23-35.

Bang W., Nikov I., Delmas H. and Bascoul A. (1998). Gas-Liquid mass transfer in a new three-phase stirred airlift reactor. *Journal Chemical Technology Biotechnology*, **72**, 137-142.

Beun J.J., Hendriks A., Van Loosdrecht M.C.M., Morgenroth E., Wilderer P.A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research,* **33** (10), 2283-2290.

Biedermann A. (1994). Scherbeanspruchung in Bioreaktoren. Dissertation Universität Köln.

Bücher K. (1993). Scherbeanspruchung in Bioreaktoren. Master Thesis Universität Pader-born.

Buys B.R., Mosquera-Corral A., Sánchez M. and Méndez R. (2000). Development and application of a denitrification test based on gas production. *Water Science and Technology,* **41**, 113-120.

Casas-López J.L., Sánchez-Pérez J.A., Fernández-Sevilla R., Rodríguez-Porcel E.M. and Chisti Y. (2005). Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus. Journal of Biotechnology,* **116**, 61-77.

Chang H.T., Rittmann B.E., Amar D.R., Ehrlinger O. and lesty Y. (1991). Biofilm detachment mechanisms in a liquid fluidized bed. *Biotechnology and Bioengineering,* **38**, 499-506.

Chen M.J., Zhang Z. and Bott T.R. (1998). Direct measurement of the adhesive strength of biofilms in pipes by micromanipulation. *Biotechnology Techniques,* **12**, 875-880.

Chisti Y. (2000). Animal-cell damage in sparged bioreactors. *Trends in Bitechnology,* **18**, 421-431.

Cirpus,I.E.Y., de Been,M., Op den Camp,H.J.M., Strous,M., Le Paslier,D., Kuenen,G.J., and Jetten,M.S.M. (2005) A new soluble 10 kDa monoheme cytochrome c-552 from the anammox bacterium Candidatus "Kuenenia stuttgartiensis". *Fems Microbiology Letters,* **252** (2), 273-278.

Dapena-Mora A., Campos J.L., Mosquera-Corral A., Jetten M.S.M. and Méndez R. (2004a). Stability of the Anammox process in a gas-lift reactor and a SBR. *Journal of Biotechnology,* **110**, 159-170.

Dapena- Mora A., Arrojo B., Campos J.L., Mosquera-Corral A. and Méndez R. (2004b). Improvement of the settling properties of Anammox sludge in an SBR. *Journal of Chemical Technology and Biotechnology,* **79** (12*),* 1412-1420.

Dapena-Mora A., Van Hulle S. W.H., Campos J.L., Méndez R. Vanrolleghem P.A. and Jetten M. (2004c). Enrichment of Anammox biomass from municipal activated sludge: experimental and modelling results. *Journal of Chemical Technology and Biotechnology,* **79**, 1421-1428.

Dapena-Mora A., Campos J.L., Mosquera-Corral A. and Méndez R. (2006). Anammox process for nitrogen removal from anaerobically digested fish canning effluents. *Water Science and Technology*, **53** (12), 265-274.

Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, **22**, 434-444.

Gjaltema A., van Loosdrecht M.C.M. and Heijnen J.J.(1997). Abrasion of suspension biofilm pellets in airlift reactors: effect of particle size. *Biotechnology and Bioengineering*, **55**, 206-215.

Guven D., Dapena A., Kartal B., Schmid M.C., Maas B., Pas-Schoonen K., Sozen S., Mendez R., Op den Camp H.J.M., Jetten M.S.M., Strous M. and Schmidt I. (2005). Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria. *Applied and Environmental Microbiology*, **71**(2), 1066-1071.

Henzler H.J. (2000). Particle stress in bioreactors. *Advances in Biochemical Engineering and Biotechnoloy,* **67**, 36-82.

Jiménez B., Noyola A., Capdeville V., Roustan M. and Faup G. (1988) Dextran Blue coulorant as a reliable tracer in submerged filters. *Water Research,* **22**, 1253-1257.

Jüsten P., Paul G.C., Nienow A.W. and Thomas C.R. (1998). Dependence of *Penicillium chrysogenum* growth, morphology, vacuolation and productivity in fed-batch fermentations on impeller type and agitation intensity. *Biotechnology and Bioengineering,* **59**, 762-775.

Klapwijk A., Jol C. and Donker H.J.G.W. (1979). The application of an upflow reactor in the denitrification step of biological sewage purification. *Water Research,* **13**, 1009-1015.

Klapwijk A., van der Hoeven J.C.M. and Lettinga G. (1981). Biological denitrification in an upflow sludge blanket reactor. *Water Research,* **15**, 1-6.

Kwok W.K., Picioreanu C., Ong S.L., van Loosdrecht M.C. M., Ng W.j. and Heijnen J.J. (1998). Influence of biomass production and detachment forces on biofilm structures in a biofilm airlif suspension reactor. *Biotechnology and Bioengineering,* **58**, 400-407.

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

Liu Y, Xu H.L., Yang S.F. and Tay J. H. (2003). Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. *Water Research,* **37**, 661-673.

Liu Y. and Tay J.H. (2004). State of the art of biogranulation technology from wastewater treatment. *Biotechnology Advances,* **22**, 533-563.

Mohan S.B., Schmid M., Jetten M. and Cole J. (2004). Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. *Fems Microbiology Ecology,* **49** (3), 433-443.

Mosquera-Corral A., Vázquez-Padín J.R., Arrojo B., Campos J.L. and Méndez R. (2005). Nitrifying granular sludge in a sequencing batch reactor. *In: Aerobic Granular Sludge. Water and Environmental Management Series.* IWA Publishing. Munich, (63-70).

Neef A., Amann R., Schlesner H. and Schleifer K.-H. (1998). Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology*, **144**, 3257-3266.

Noyola A. and Moreno G. (1994). Granule production form raw waste activated sludge. *Water Science and Technology,* **30**, 339-346.

Ohashi A. and Harada H. (1994). Adhesion strength of biofilm developed in an attached-growth reactor. *Water Science and Technology,* **29**,10

O'Flaherty V., Lens P.N., de Beer D. and Colleran. E. (1997) Effect of feed composition and upflow velocity on aggregate characteristics in anaerobic upflow reactors. *Appied Microbiology and Biotechnology,* **47**, 102-107.

Pinho F.T., Piqueiro F.M., Proenca M.F. and Santos A.M. (1997). Power and mean flow characteristics in mixing vessels agitated by hyperboloid stirrer. *Canadian Journal of Chemical Engineering,* **75** (5), 832-842.

Sánchez-Mirón A., Cerón M.C., Contreras A., García F., Molina E. and Chisti Y. (2003). Shear stress tolerance and biochemical characterization of Phaedactylum tricornutum in quasi steady-state continuous culture in outdoor photobioreactors. *Biochemical Engineering Journal*, **16**, 287-297.

Schmid M., Schmitz-Esser S., Jetten M. and Wagner M. (2001). 16S-23rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental Microbiology,* **3** (7), 450-459.

Schmidt I., Hermelink C., van de Pas-Schoonen K., Strous M., Camp H.J., Kuenen J.G.and Jetten M.S.M. (2002). Anaerobic ammonia oxidation in the presence of nitrogen oxides (NOx) by two different lithotrophs. *Applied Environmental Microbiology,* **68** (11), 5351-5357.

Schmid, M., K. Walsh, R. Webb, W. I. C. Rijpstra, K. van de Pas Schoonen, M. J. Verbruggen, T. Hill, B. Moffett, J. A. Fuerst, S. Schouten, J. S. S. Damsté, J. Harris , P. Shaw, M. Jetten, and M. Strous (2003). *Candidatus "Scalindua brodae", spec. nov., Candidatus "Scalindua wagneri", spec. nov.,* two new species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology*, **26**, 529–538.

Schmid M, Mass B., Dapena A., *et al*. (2005). Biomarkers for In situ Detection of Anaerobic Ammonium-Oxidizing (Anammox) bacteria. Minireview. *Applied and Environmental Microbiology*, **71** (4), 1677-1684.

Sinninghe-Damste J.S., Strous M., Rijpstra W.I.C., Hopmans E.C., Geenevasen J.A.J., van Duin A.C.T., van Niftrik L.A. and Jetten M.S.M. (2002). Linearly concatenated cyclobutane lipids form a dense bacterial membrane. *Nature* **419**, 708-712.

Sliekers A., Third K.A., Abma W., Kuenen J.G. and Jetten M.S.M. (2003). CANON and Anammox in a gas-lift reactor. *FEMS Microbiology Letters,* **218**, 339-344.

Strous M., Heijnen J.J., Kuenen J.G. and Jetten M.S.M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology,* **50**, 589-596.

Strous M., Kuenen J.G. and Jetten M.S.M. (1999). Key physiology of anaerobic ammonium oxidation. *Applied Environmental Microbiology,* **65**, 3248-3250.

Strous M., Kuenen J.G., Fuerest J.A., Wagner M. and Jetten M.S.M. (2002). The Anammox case- A new experimental manifesto for microbiological eco-physiology. *Antonie van Leuwenhoek.* **81**, 693-702.

Tay J-H, Liu Q-S and Liu Y. (2001). The effects of shear force on the formation, structure and metabolism of aerobic granules. *Applied Microbiology and Biotechnology,* **57**, 227-233.

Tay J.H., Liu Q.S. and Liu Y.(2004). The effect of upflow air velocity on the structure of aerobic granules cultivated in a sequencing batch reactor. *Water Science and Technology,* **49** (11-12), 35-40.

Tijhuis L., van Loosdrecht M.C.M. and Heijenen J.J.(1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioengineering*, **44**, 595-608.

Tijhuis L., Hijman B., van Loosdrecht M.C.M. and Heijnen J.J. (1996). Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. *Applied Microbiology and Biotechnology,* **45**, 7-17.

van Benthum W.A.J.,Garrido-Fernández J.M., Tijhuis L., van Loosdrecht M.C.M.. and Heijnen J.J. (1996). Formation and detachment of biofilms and granules in a nitrifying biofilm airlift suspension reactor. *Biotechnology Progress,* **12**, 764-772.

van Benthum W.A.J., van Loosdrecht M.C.M.. and Heijnen J.J. (1997). Process design for nitrogen removal using nitrifying biofilm and denitrifying suspended growth in a biofilm airlift suspension reactor. *Water Science and Technology,* **36**, 119-128.

van de Graaf A. A., de Bruijn P., Robertson L.A., Jetten M.S.M. and Kuenen J. G. (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology (UK)* **142**, 2187-2196.

6.7 NOMENCLATURE

a	=	Impeller area (m^2)
d	=	Impeller diameter (m)
D	=	Reactor diameter (m)
h	=	Impeller height (m)
H	=	Reactor height (m)
HRT	=	Hydraulic Retention Time (d)
l	=	Stirrer length (m)
N	=	Rotating speed (rpm)
Ne	=	Newton number
NLR	=	Nitrogen Loading Rate (g N/(L·d))
P	=	Input power (kW)
P/V	=	Specific power (kW/m^3)
Re	=	Reynolds number
SAA	=	Specific Anammox Activity ($g\ N\cdot(g\ VSS\cdot d)^{-1}$)
SBR	=	Sequencing Batch Reactor
SEM	=	Scan Electron Microscope
SRT	=	Solids Retention Time (d)
SVI	=	Sludge Volumetric Index (mL/g VSS)
TSS	=	Total Suspended Solids (g/L)
UASB	=	Upflow Anaerobic Sludge Blanket
V	=	Reactor volume (L)
VSS	=	Volatile Suspended Solids (g/L)
w	=	Blade width (m)
μ	=	Fluid viscosity (kg/(m·s))
ρ	=	Fluid density (kg/m)

Chapter 7

A membrane coupled to a sequencing batch reactor for water reuse and removal of coliform bacteria[1]

Summary

Wastewater reclamation was studied by using a lab-scale biological reactor and an external filtration membrane coupled in series. The use of the membrane enhanced the quality of the produced effluent from the biological reactor in terms of suspended solids and presence of indicator bacteria. Partial removal of faecal coliforms and *Escherichia Coli* was observed in the effluent of a sequencing bacth reactor (SBR), previous to filtration by the membrane. The use of the membrane ensures a full removal of the indicator bacteria in the final permeate. More than 95% of the organic matter, suspended solids, and coliform bacteria were successfully removed.

The operation and behaviour of internal submerged membranes in two different bioreactors was an additional objective. For this reason, two configurations: (i) a membrane coupled to a SBR (MSBR); and (ii) a membrane continuous bioreactor (MBR) was used during the study. Particular attention was focussed on fouling and hydraulic operational conditions of the membranes. Fouling problems of the membrane were reduced by maintaining turbulent conditions and by operating at sub-critical flux.

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7.1. Introduction

Europe has plenty of water resources compared to other regions of the world, and water has long been considered as an inexhaustible public commodity. This position has, however, been challenged in the last decades by growing water stress, both in terms of water scarcity and quality deterioration (Bixio *et al.*, 2006). Approximately half of the European countries, representing almost 70% of the population, are facing nowadays problems with the water supply (Hochstrat and Wintgens, 2003).

Existing municipal wastewater treatment plants have to meet increasingly stringent discharge limits for organic matter (BOD_5, COD) and suspended solids (SS) content. New regulations also impose nitrogen and phosphorus discharge limited levels or regulated removal efficiencies and determined bacteriological quality, especially in environmentally sensitive areas (Countil Directive, 1975, 1991). To abide by the new regulations, plant up-grading to meet the standards is often necessary.

The optimisation of existing conventional processes and facilities, while representing the first logical response to meet this challenge will be limited to the maximum efficiency technically and economically achievable by such conventional processes (limits often associated with the performance of the secondary clarifier). Innovative up-grading schemes for sewage treatment plants are emerging in response to this challenge. The use of immersed membranes as biomass separators in secondary treatment systems is an approach which holds interesting promises in this context; and, in the case of a sequencing batch reactor (SBR), the use of an external membrane module coupled to the system could be a suitable technology to achieve high quality effluents (Buisson *et al.*, 1998).

SBR processes offer several advantages over other types of activated sludge reactors. In particular, the hallmark of the SBR design is its inherent flexibility of cyclic phasing. The cycle format can be easily modified at any time to offset changes in process conditions, influent characteristics or treatment objectives (Epa, 1999; Pavelj *et al.*, 2001; Pochana and Keller, 1999; Kang *et al.*, 2003). However, a critical aspect of the SBR technology could be the poor clarification associated with effluent turbidity, which occurs in some operational conditions.

The combination of a membrane system with a SBR provides procedural advantages for both processes. The use of membranes can reduce the operation period since the membrane separation of the solids from the effluent requires no settling phase and clear-water can be extracted even during the mixing phase (Kang *et al*., 2003). In addition, the separation of biological sludge by means of a membrane leads to complete retention of biomass resulting in a high mixed liquor suspended solids concentration inside the reactor. This allows a very high treatment capacity for a membrane-coupled sequencing batch reactor (MSBR) (Krampe and Krauth, 2000). In a MSBR system, it would be very important, and also difficult to select the most appropriate SBR phase in which the membrane filtration could be performed at its best. The reason for this relies on the many types of SBR systems currently in use which operate in different operation modes such as: continuous influent/time based, non-continuous influent/time based, volume based, and intermittent cycle system, and various other system modifications (Banas *et al*., 1999; Schleypen *et al*., 1997; Ketchum, 1997; Irvine *et al.,* 1997). Although combination of membrane filtration and SBR should be reengineered to get a MSBR of good performance, the efficiency of the membrane process in MSBR would certainly be dependent on the physicochemical and biological conditions of each SBR phase as well as the type and cycle format of the SBR.

Regarding the operation of the membrane certain aspects must be taken into account to achieve optimal operation conditions and are basically related to the fouling problems associated to the solids content in the liquid media. The presence of fouling provokes problems of operation and reduces the achievable flow rate of liquid through the membrane system, decreasing at the same time the quality of the produced effluent (Wei *et al*., 2006; Cho and Fane, 2002; Ognier *et al.,* 2004; Tyszler *et al*., 2006; Judd, 2004). For this reason the operation of the membrane must be followed by knowing the values of certain operational parameters such as the critical flux. The concept of the critical flux has been introduced recently by a number of authors providing theoretical and experimental evidence. Fields *et al.* (1995) and Howell (1995) stated that “there exists one flux below which a decline of flux with time does not occur; above this flux, fouling is observed. This flux is termed as the critical flux and its value

depends on the hydrodynamics and probably also on other variables". This flux should be equivalent to the corresponding clean water flux at the same transmembrane pressure (TMP). Theoretical calculations made by Bacchin *et al.* (1995) suggested that the critical flux depends strongly on the particle size. For small particles of the order of 0.1 μm back-diffusion away from the membrane surface is important and the critical flux depends mainly on the surface charge of particles. For particles over 1 μm, the shear-induced diffusion which lifts particles away from the membrane surface and this is critical for the membrane fouling. Fouling can be reduced by maintaining turbulent conditions, operating at sub-critical flux and selection of a suitable fouling-resistant membrane material (Gander *et al.*, 2000).

The membrane bioreactors (MBR) present a means to biologically treat high COD or BOD loaded wastewaters and also to reduce the microbial content of the wastewater.

Because membranes are an absolute barrier for bacteria and in the case of ultra filtration (UF) also for viruses, the MBR process provides a considerable level of physical disinfection (Melin *et al.*, 2006). The microfiltration membranes applied in MBRs have proven to achieve consistently high removal rates for microbiological parameters such as total coliforms, faecal coliforms and even bacteriophages. The log removal reported varied between 6-8 log for bacteria and 3-5 log scales for viruses (Stephenson *et al.*, 2000). MBR effluents were found to be compliant with the EU Bathing Water Directive (EC/160/75) including parameters such as total coliforms, faecal coliforms, *Streptococcus faecalis* and Coliphages (Günder and Krauth, 1999). Experiments conducted by Cicek *et al.* (1998) with indicator viruses MS-2, which have an approximate diameter of 25 nm and were spiked to the feed of the membrane, revealed removal percentages of 94.5%. A 5.88 log removal for bacteriophages was observed by Ueda and Horan (2000) in an MBR treating settled sewage and partially attributed to retention by the membrane and adsorption to activated sludge. A 6.86 log removal was found for faecal coliforms. This compares favourably with a conventional activated sludge treatment plant, showing only 1.31 log reduction for bacteriophages and 2.34 log for faecal coliforms (Ueda and Horan *et al.*, 2000).

The favourable microbiological quality of the effluent of MBRs is a major factor in their frequent selection for water reuse, even if full disinfection can not be expected, particularly considering the distribution and storage components of a full-scale system, which can be prone to re-growth of microorganisms and contamination from various sources (Coté *et al*., 1998). The biggest MBR system of the world up to date, the municipal wastewater treatment in Kaarst, Germany, features online particles turbidity measurement in the main effluent discharge line. Periodically integrity testing can be performed with full-scale modules with bubble point or pressure-decay tests (Melin *et al*., 2006).

Regarding the applicability of the MBR systems for water reuse it has to be taken into account that once governments are convinced of the need of water reuse, it is not always easy to obtain a permit for the reuse of reclaimed water, and this despite the European Unions' wide encouragement to reuse wastewater treatment effluents. For several member states one of the major problems is the lack of clear criteria on when to reuse and on quality standards (Bixio *et al*., 2006).

Despite the fact that no guidelines or regulations yet exist at the European Union level, several member states or autonomy regions have now published their own standards or regulations. For example, in Italy with Decree of Environmental Ministry 185/2003 the quality requirements were defined for the three water reuse categories: agriculture, non-potable urban and industrial. At the same time a reduction of microbial load is necessary for water reuse in agriculture, as stated in the Italian legislation, which indicates a limit of 10 CFU/100mL for *E. Coli* (Decree, 185/2003). In Spain, in 1985 the Government indicated water reuse as a possibility, but no specific regulations followed. A draft legislation has been issued in 1999, with a set of standard for 14 possible applications of treated water. The proposed microbiological standards range is strongly similar to those of the California Title 22 regulations.

7.2. Objectives

The main objective of this study was the evaluation of the applicability of the coupled system comprising a bioreactor and a membrane system for the removal of organic matter and faecal coliforms and *Escherichia Coli* in order to obtain water suitable for reuse.

Two different configurations were tested using two different bioreactors: (i) a membrane coupled to a SBR (MSBR) and (ii) a membrane continuous bioreactor (MBR) was studied with particular attention paid to the fouling and the hydraulic conditions.

7.3. Materials and methods

7.3.1. Experimental set-up

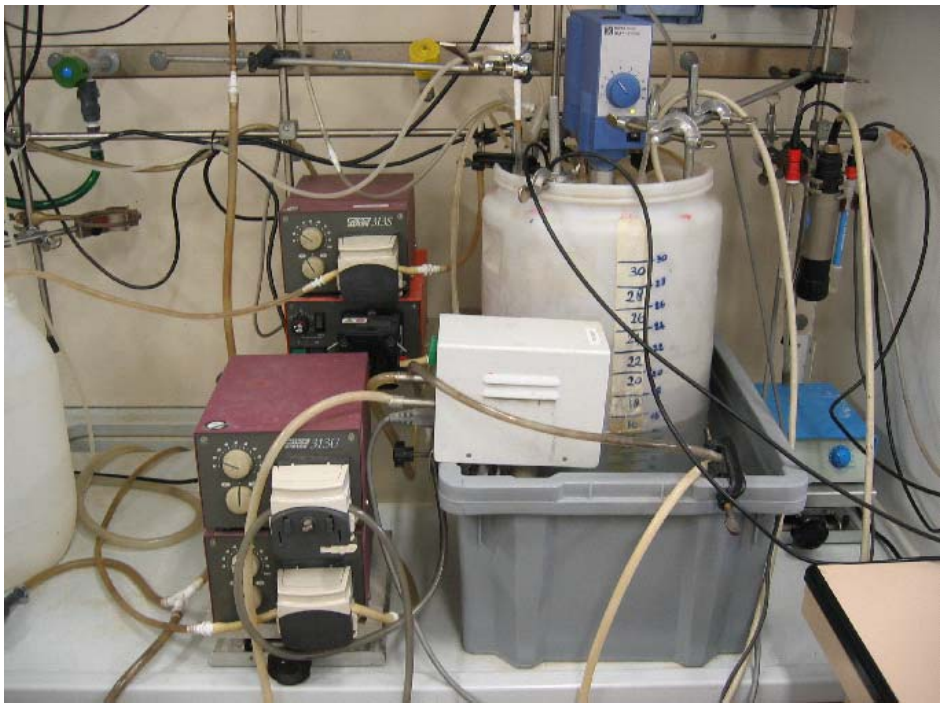
A. Membrane-coupled SBR

The laboratory-scale SBR reactor was a cylindrical vessel with a working volume of 20 L. Peristaltic pumps were used for feed, discharge of the effluent and biomass purging. During the oxic phase, oxygen was supplied by an air blower (mass transfer coefficient, K_La = 0.2 min^{-1}). Mechanical mixing was supplied during both the oxic and anoxic phase at a rotating speed of 400 rpm. The actions of the pumps, aeration system and stirrer were controlled by four timers (Figure 7.1).

The reactor was equipped with a data acquisition system MARTINA (Ficara *et al.*, 2000) (Multiple Analyse Reprogrammable TItratioN Analyser), Spes scrl, Fabriano (AN)) with the following probes:

- ORP probe (InLab 501, Mettler Toledo- Greifensee, Swithzerland).
- Dissolved oxygen probe (COS3S, Endress-Hauser, Reinach,, Switzerland).
- pH electrode (InLab 412, Mettler Toledo Greifensee, Switzerland).
- Temperature probe (Pt100, TRM).

Data were acquired each 20 seconds by means of a data acquisition system.



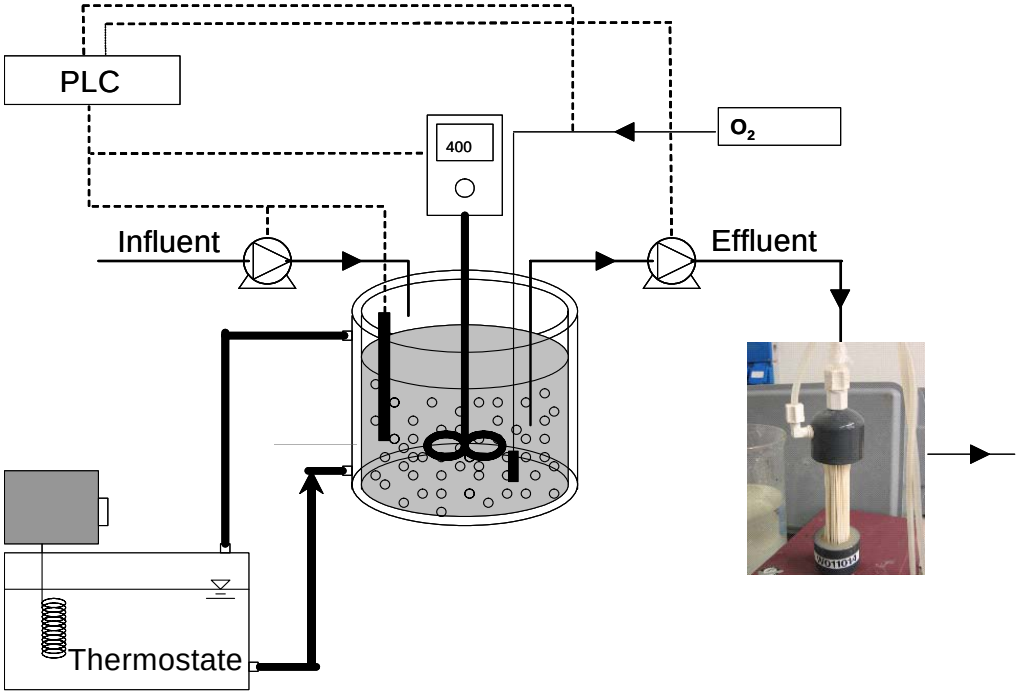


Figure 7.1. Sequencing Batch Reactor and membrane.

Temperature was maintained at 25 ºC by a thermostatic bath, while pH varied between 7.2 and 8.3.

The effluent from the SBR was pumped for filtration with a hollow fibre membrane module ZW-1 (Zenon) with a pore size of 0.2 μm and an effective surface area of 0.093 m^2. The external diameter of each fibre was 1.8 mm while the internal diameter was 0.5-1.0 mm. The membrane was 50 mm wide and 175 mm long (Figure 7.2).

The ZW-1 module comes with an extended aeration tube that is also used to attach the module to the support bracket to hold it in place vertically. It has two holes on the top header: one for the permeate and one for pressure measurement. The permeate is drawn only from the top header. The central aeration tube supplies air to the bottom header where air diffusers are located. The ZW-1 module was connected to a vacuometer in order to measure the Trans-Membrane Pressure (TMP).

Figure 7.2. Membrane module (Zenon ZW-1).

B. Hydraulic conditions

Two configurations of the collocation of the membrane were compared: (i) a membrane ZW-1 coupled to a SBR with intermittent permeate extraction (MSBR) (Figure 7.3) and (ii) a membrane bioreactor with continuous permeate extraction (MBR) (Figure 7.4). Two laboratory-scale SBRs with a total volume of 5 L and a working volume of 4 L were used. In the first configuration, the system was operated with the same reaction phase as the 20 L SBR described above, but no sedimentation was performed since the membrane module was used for effluent extraction. The second reactor was operated as a MBR constantly aerated and with constant permeate extraction.

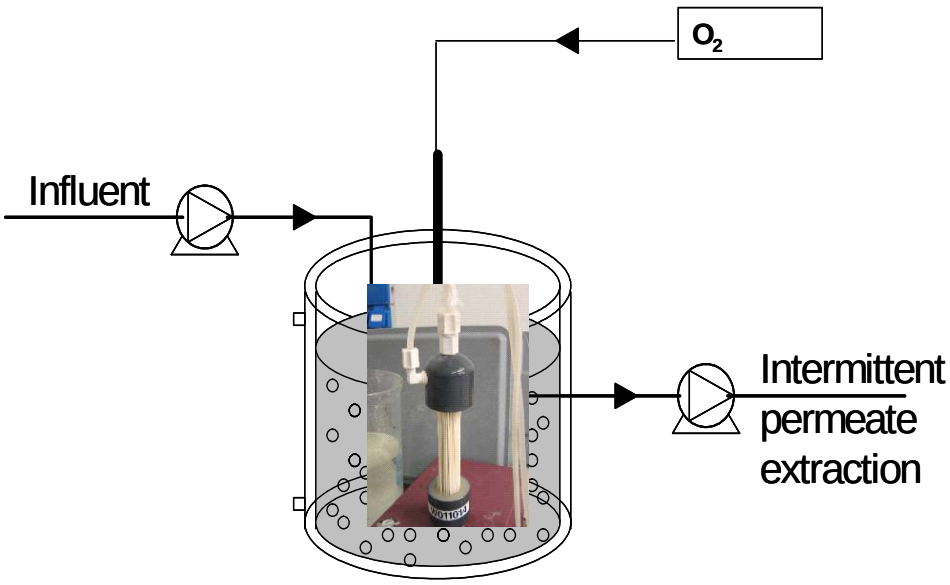


Figure 7.3. Membrane coupled to a SBR with intermittent permeate extraction (MSBR).

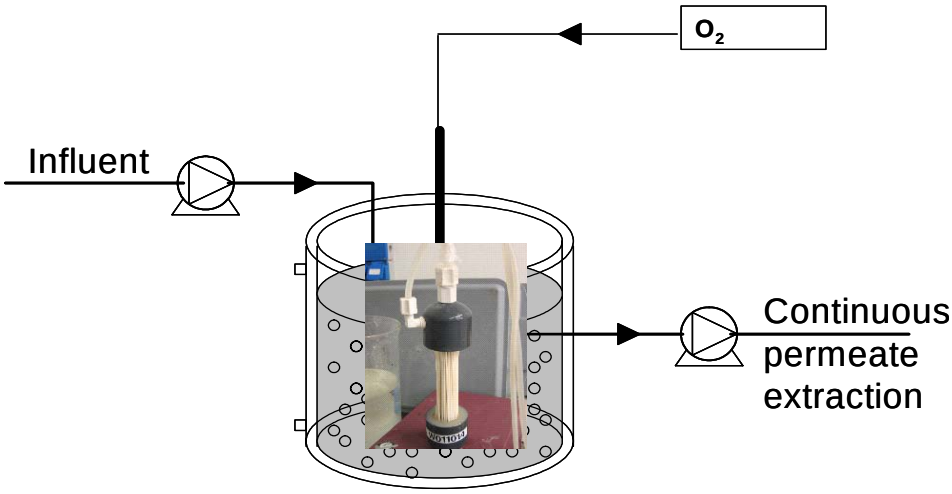


Figure 7.4. Membrane Bioreactor (MBR).

7.3.2. Feeding media

Initially, the reactor was fed with a synthetic medium made of peptone, meat extract and salts (COD = 600 mg/L, Ntot = 75 mg/L, Ptot = 11.4 mg/L) (Table 7.1) and the trace solution according to Larsen and Harremoes (1994) (Table 7.2). The microbial inoculum came from a primary treated urban wastewater (Table 7.3) which was added to the synthetic feed (1/10 v/v). Finally, some cycles were performed feeding urban wastewater to assess the membrane efficiency on undiluted real wastewater.

Table 7.1. Composition of synthetic medium used to feed the SBR.

Compounds	**Values (g/L, except the traces solution)**
Peptone	0.457
Meat extract	0.236
NaCl	0.015
$CaCl_2 \cdot 2H_2O$	0.012
$MgSO_4 \cdot 7H_2O$	0.0045
K_2HPO_4	0.06
Traces solution	0.10 mL/L

Table 7.2. Composition of traces solution.

Compounds	Values (g/L)
$CuCl_2*2H_2O$	0.228
$CoCl_2*6H_2O$	0.317
$(NH_4)_6Mo_7O_{24}*4H_2O$	0.527
$MnCl_2*4H_2O$	0.527
$Na_2B_4O_7*10H_2O$	0.127
$ZnCl_2$	0.363
$FeSO_4*7H_2O$	3.7

Table 7.3. Average composition of the urban wastewater treatment plant of Pero (Milano).

Compounds	Values (mg/L, except pH)
pH	7.73
TSS	86.79
BOD_5	99.77
COD	266.74
Ntot	32.36
$N\text{-}NH_4^+$	18.77
Ptot	3.79
Al	1.56
Cr	0.10
Fe	0.94
Pb	0.01

7.3.3. Strategy of operation

The reactor was operated during the two modes of operation with a hydraulic retention time (HRT) of 1.25 days. The SBR was operated in cycles of 6 hours including a feeding phase of 30 min, a reaction phase of 300 min (90 anoxic, 210 aerobic), a settling period of 37 min and an effluent withdrawal period of 23 min (Figure 7.5).

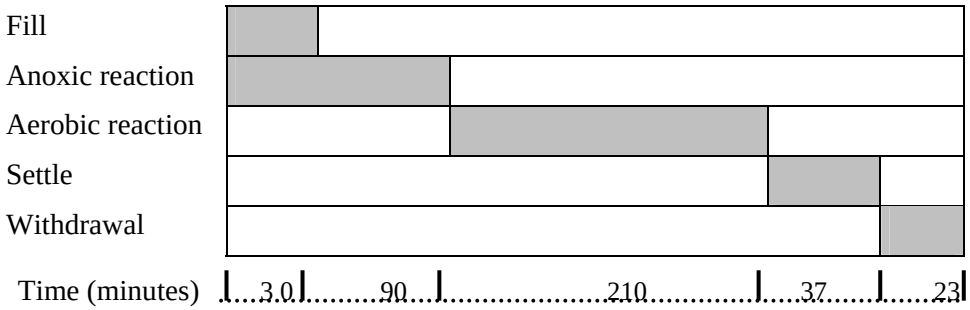


Figure 7.5. Cycle distribution for the SBRs operation.

7.3.4. Analytical methods

The pH, nitrate, ammonia, total nitrogen, volatile suspended solids (VSS), total suspended solids (TSS) and COD were determined according to Standard Methods (APHA, 1999) as described in Chapter 2.

Microbiologic analysis

Faecal coliforms and *Escherichia Coli* were measured by a membrane filtration technique combined with selective growth on C-EC Agar. Results are expressed as Coliform Forming Units (CFU) in 100 mL/sample (Isra-Cnr, 1994) (Chapter 2). *E. Coli* was enumerated from faecal coliforms by using the wood Lamp.

7.3.5. Determination of critical and maximum flux

Critical and maximum fluxes were determined by monitoring the TMP according to the procedure suggested by Kwon (Kwon *et al*., 2000). This method is based on the increase in TMP required to maintain a constant permeate flux. The TMP increases during the constant permeate flux operation in order to compensate the increase in the resistance to permeation. The critical flux is the flux below which there is no presence of this increase in resistance to permeation (i.e. the TMP is constant with time).

Thus, there will be no increase in the TMP with time if no or negligible membrane fouling occurs. On the other hand, the increase in TMP implies that the cake layer formed by deposited particles caused significant resistance to permeate flow (fouling effect).

7.4. Results and discussion

7.4.1 Membrane-coupled SBR

During the entire experimental period, the SBR effluent was characterised by: COD < 50 mg/L, TKN < 5 mg N/l, NO_3^- < 20 mg N/L. The COD removal efficiency in the SBR was 95%. The concentration of suspended solids in the effluent of the SBR was lower than 50 mg TSS/L and a complete removal was achieved after filtration with the ZW-1 membrane module.

The operational period can be divided into two periods according to the type of SBR feeding:

i) 10 % urban wastewater in the feed (50 days of operation).

In the SBR influent, faecal coliforms were in the range 7×10^4- 1.5×10^5 CFU/100 mL and 3.7×10^4 -1×10^5 CFU/100 mL as *E.Coli*. In the effluent of the SBR, faecal coliform were around 4×10^3-1.0×10^5 CFU/100 mL and *E.Coli* around 3×10^2-4.3×10^4 CFU/100 mL, while no faecal coliform nor *E-Coli* were found in the permeate (Figure 7.6 and 7.7).

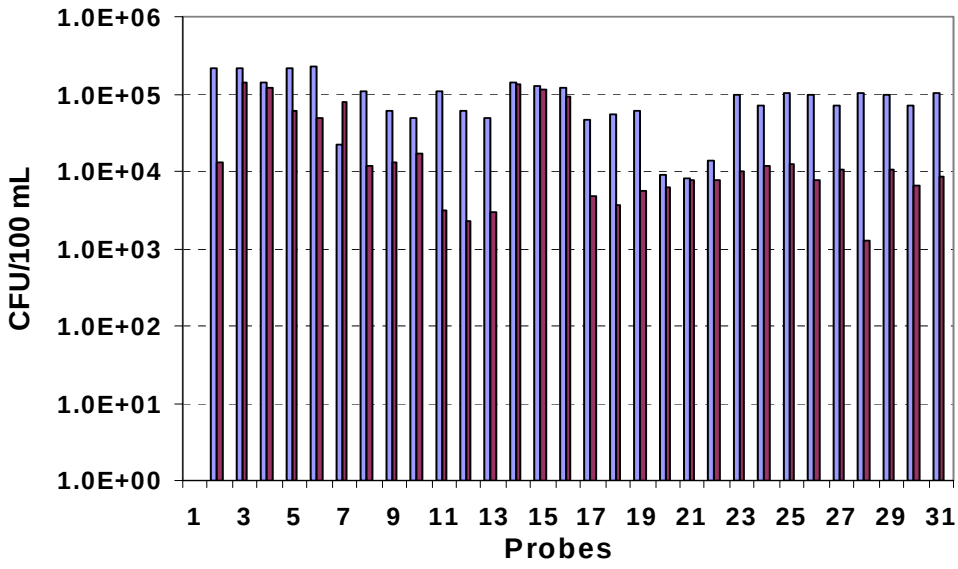


Figure 7.6. Evolution of the Faecal Coliforms in the system with 10 % urban wastewater in the feeding (■ influent SBR, ■ effluent SBR previously to the filtration).

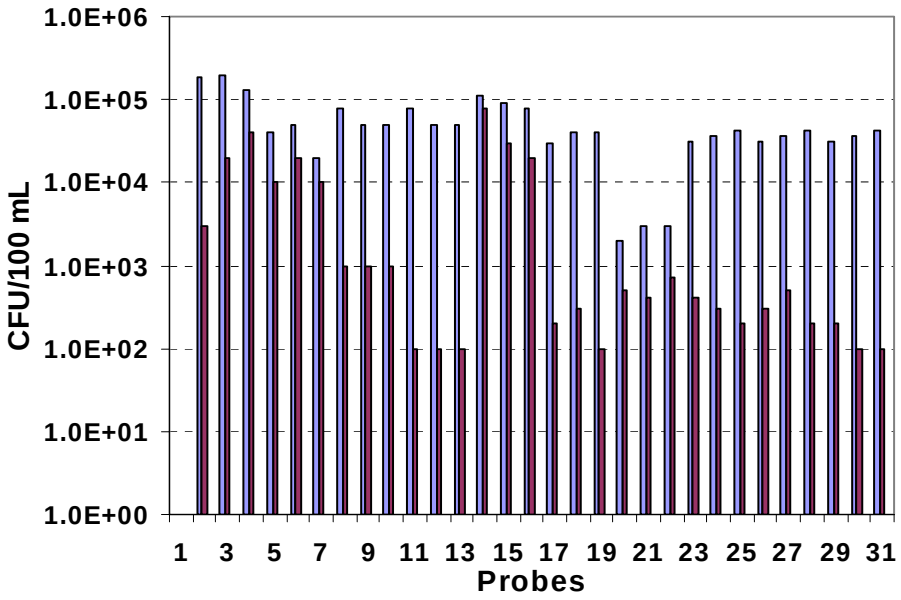


Figure 7.7. Evolution of the E.Coli in the system with 10 % urban wastewater in the feeding (■ influent SBR, ■ effluent SBR previously to the filtration).

ii) 100% urban wastewater in the feeding (10 days of operation).

Faecal coliforms were in the range 1.2×10^{6}- 1.8×10^{6} CFU/100 mL in the SBR influent and 4.0×10^{3} -1.0×10^{4} CFU/100 mL in the SBR effluent (Figure 7.8), while *E.-Coli* were 1.0×10^{6}-1.4×10^{6} CFU/100 mL and 2.0×10^{3}-4.1×10^{3} CFU/100 mL for the influent and effluent, respectively (Figure 7.9). Neither faecal coliform nor *E.Coli* were found in the permeate.

These results are in agreement with those reported in previous attempts to apply membrane filtration on raw/biologically-treated domestic sewage. Ueda and Hata (1999) have operated a MBR with gravitational filtration using a pilot-scale plant and raw domestic wastewater. Treated water was filtered through flat microfiltration membrane modules (polyethylene: pore size 0.4 μm) and quality of the treated water indicated that the removal of organic matter and suspended solids was quite successful. Coliform bacteria were detected in the treated water at trace levels, due to the contamination of pipelines for the treated water. Nevertheless, a 6-log removal of coliform bacteria was achieved.

Other studies (Ueda *et al.*, 1996) have reported that more than 90% of organic matter, suspended solids, and coliform bacteria were successfully removed from a domestic sewage using a hollow fibre membrane. Moreover, an activated sludge system with cross-flow membrane filtration was found to remove bacteria and particular solids to concentrations fitting reuse requirements. High COD and N removal efficiencies (about 98%) were also achieved (Kishino *et al.*, 1996).

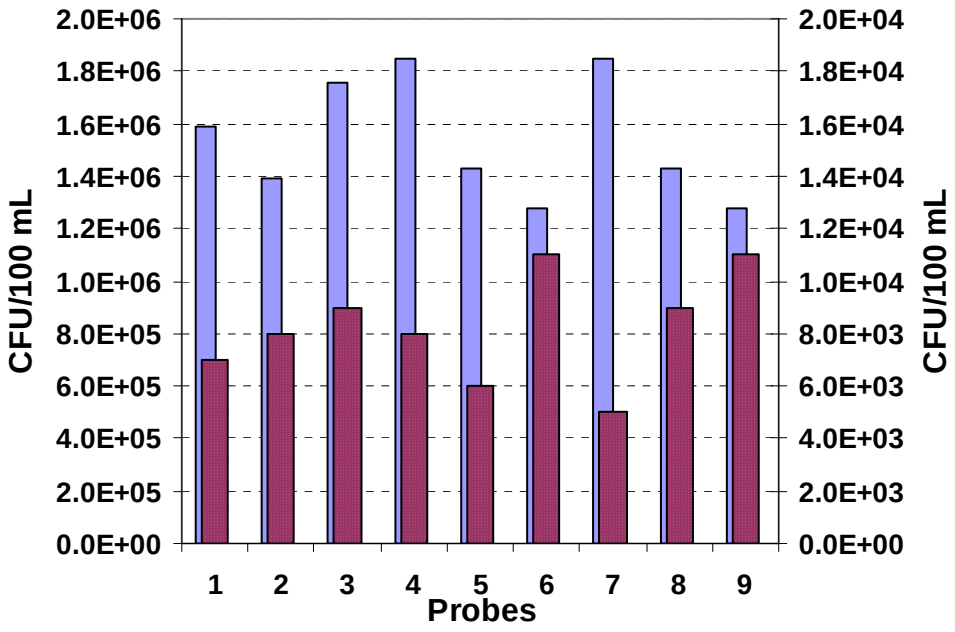


Figure 7.8. Evolution of the Faecal Coliforms in the system with 100 % urban wastewater in the feeding (■ influent SBR, left axis, ■ effluent SBR previously to the filtration, right axis).

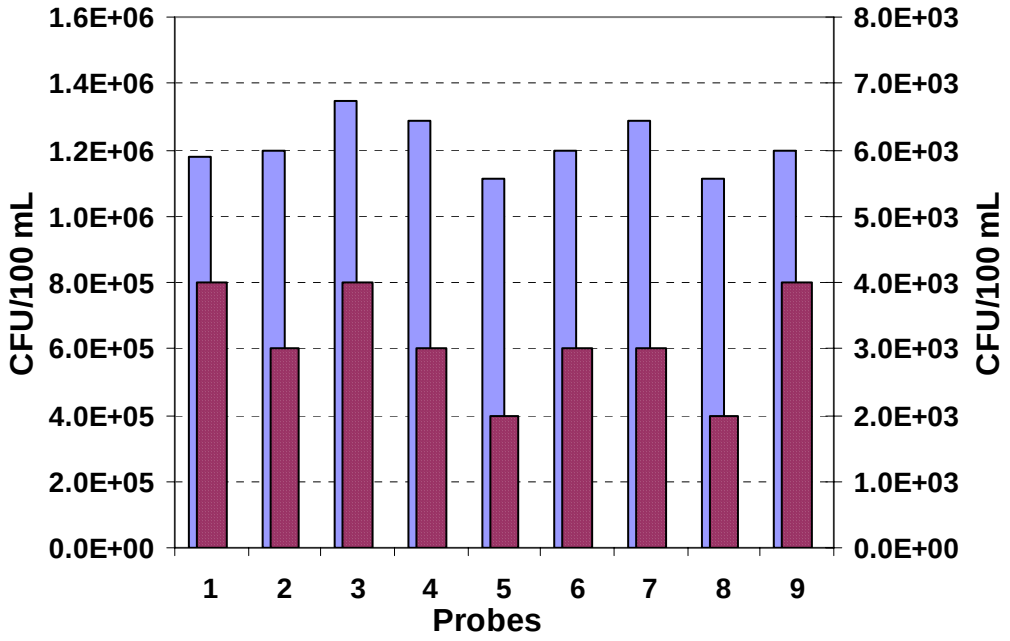


Figure 7.9. Evolution of the E-Coli in the system with 100 % urban wastewater in the feeding (■ influent SBR left axis, ■ effluent SBR previously to the filtration, right axis).

7.4.2 Determination of critical flux and maximum flux

The hollow fibre membrane was characterized to determine its response to the different applied permeate fluxes and to the solids concentration in the liquid media. Tap water was used to research the effect of the permeate flux. The results indicate a linear dependence of the flux with the TMP (Figure 7.10). The TMP increased when the flux was also increased.

The influence of the VSS concentration on the TMP was also studied by means of experiments performed in different periods with different solids concentrations in the reactor. The obtained results shown that when operating with a fixed flux value the TMP increased at the same time the concentration of solids increased (Figure 7.11).

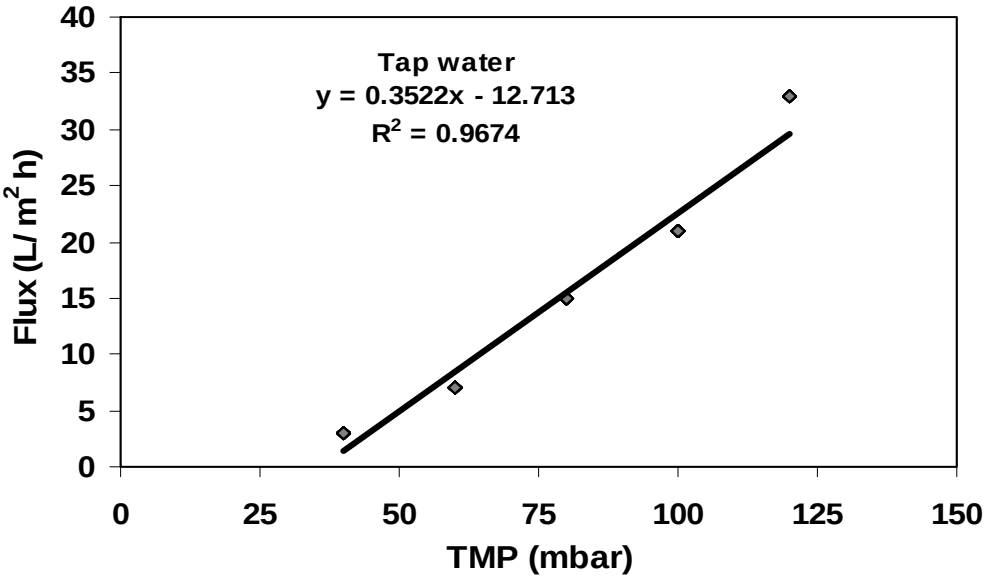


Figure 7.10. Characterization hollow fibre Zenon ZW-1.

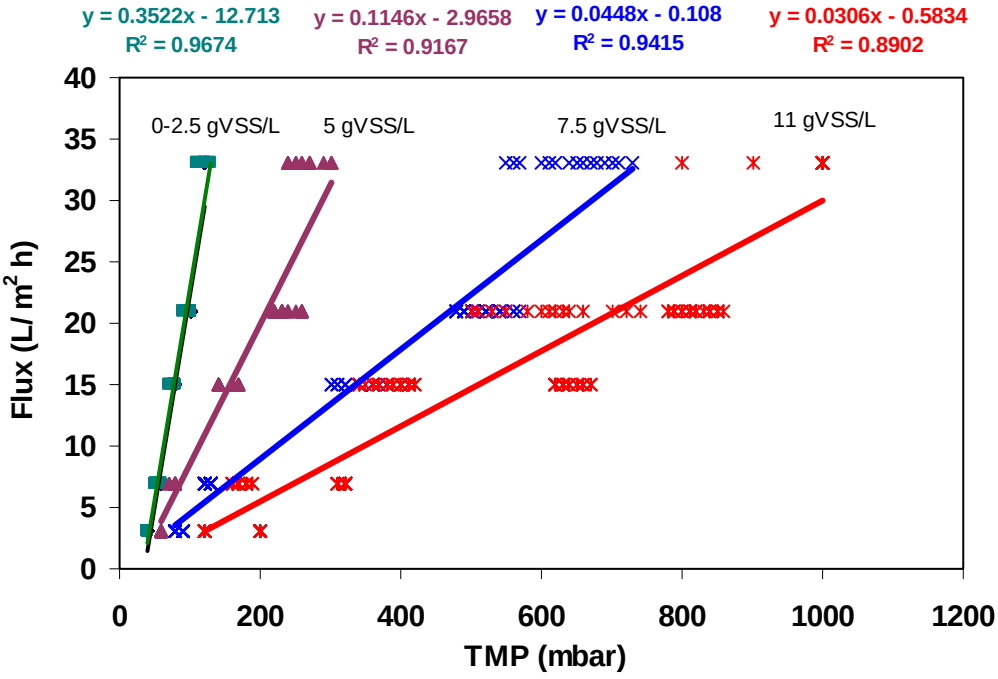


Figure 7.11. Influence of the VSS on the TMP.

In order to obtain the optimal conditions of operation of the membrane, the critical flux and maximum flux were determined for different VSS concentrations and are reported in Figure 7.12. At VSS concentrations in the range 0 and 2.5 g VSS/L the critical flux was not reached at the maximum flow tested (35 L/(h·m^2)) as the TMP was maintained constant. In the case of VSS concentrations of 5.0-7.5 g VSS/L, the critical flux was reached at 20-25 L/(h·m^2). Higher values of the TMP increased with the operation time. The value of critical and maximum flux was below 10 L/(h·m^2) at VSS concentration of 11 g VSS/L These data should be considered as comparative and not absolute values, as, of course, the laboratory-scale conditions are not comparable to full-scale conditions where the control of fouling can be optimised.

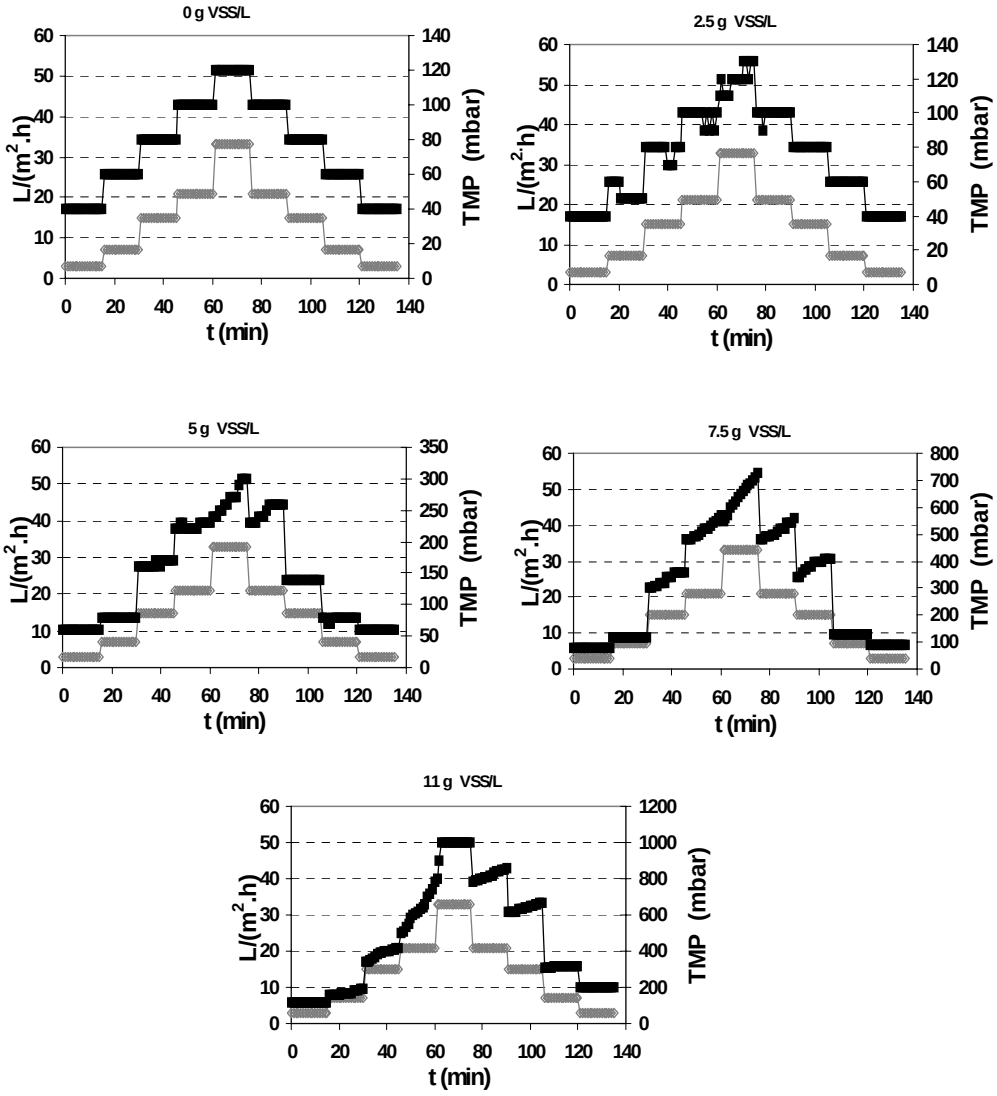


Figure 7.12. Variation of TMP with time under stepwise increments of permeate flux to different VSS concentrations (■ TMP, ■ L/(m^2.h))

7.4.3 Two configurations

Both configurations: (i) a membrane coupled to a SBR (MSBR) with intermittent permeate extraction and (ii) a membrane bioreactor (MBR) with continuous permeate extraction, were used to monitor their performances at equal daily flow permeate extraction.

The two reactors were inoculated with 2.5 g VSS/L from 20 L SBR. The main operational stages of the reactors were (Figure 7.13):

a) First period (0-10,000 min)

Although the operation of the MSBR was satisfactory during first cycles (TMP was maintained constant around 150 mbar) to a flux of 35 L/(h·m^2) with withdrawal time of 1 h/d, after 4,000 min the TMP was increased along the withdrawal time and subsequently fouling was reached. Chemical cleaning of the membrane was necessary.

The MBR was operated continually with a flow of 1.5 L/(h·m^2) and TMP was maintained constant (<50 mbar) during the entire operation, except when the flow was increased drastically.

b) Second period (10,000-20,000 min)

The withdrawal time was increased to 4 h/d, decreasing the flux to 8.5 L/(h·m^2) and the operation of the MSBR was stable (TMP = 70 mbar). The MBR was also stable at the same flux.

c) Third period (20,000-30,000 min)

The withdrawal time was maintained and the flow was increased to 28 L/(h·m^2) in the MSBR and MBR. The operation of the membranes was satisfactory. Optimal conditions were reached to operate the MSBR and the MBR.

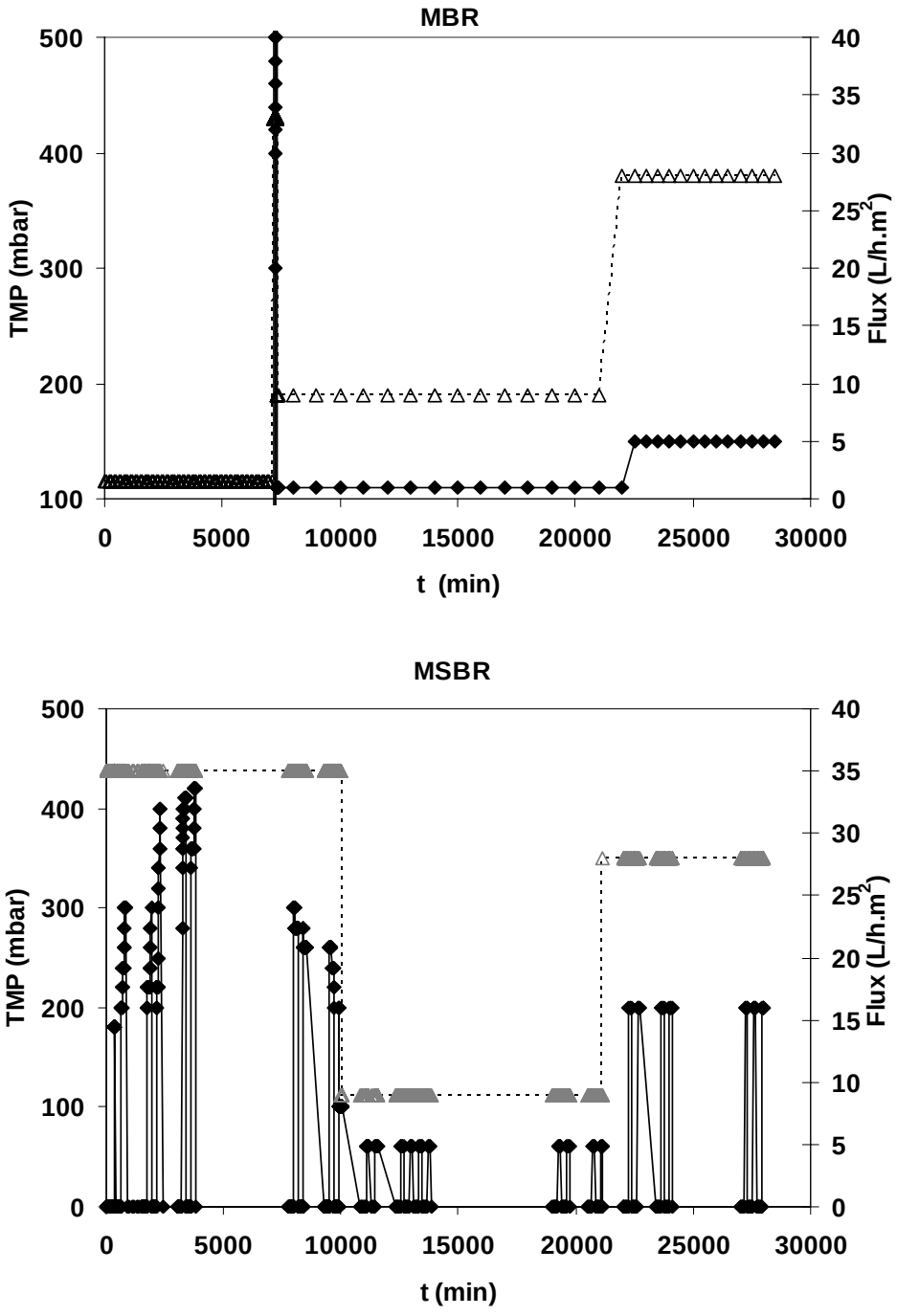


Figure 7.13. Operation of MSBR and MBR (● TMP; △ L/(m^2.h))

7.5. Conclusions

The presented work illustrates that membrane technology has a significant potential to become a key element of wastewater reclamation and reuse schemes world-wide. Membranes for municipal wastewater treatment feature advantages compared to conventional activated sludge plants in terms of effluent quality, reflected in lower values for organics, nutrients and microorganisms.

Results demonstrated that the removal efficiency of both bacteria and suspended solids by membrane filtration was 100%, suggesting that the experimented compact system (SBR+membrane filtration) could produce an effluent suitable for reuse in agriculture and could be a suitable technology for rural communities. The membrane process coupled with a SBR not only replaces the sedimentation period in the operation of a SBR but also serves as an advanced treatment unit for coliform bacteria and suspended solids, which cannot be removed completely by conventional processes.

The operation of two configurations, a membrane coupled to a SBR and a MBR, were satisfactory to operate the system to the optimal hydraulic conditions.

7.6. References

APHA-AWWA-WPCF. (1999). Standard Methods for the examination of water and wastewater. 20th Edition. Clesceri, L.S., Greenberg, A.E. & Eaton, A.D. (eds.).

Bacchin P., Aimar P. and Sanchez V. (1995). Model for colloidal fouling of membranes. *AICHE Journal*, **41** (2), 368-376.

Banas J., Plaza E., Styka W. and Trela J. (1999). SBR technology used for advanced combined municipal and tannery wastewater treatment with high receiving water standards. *Water Science and Technology*, **40** (4-5), 451-8.

Bixio D., Thoeye C., De Koning J., Joksimovic D. Savic D., Wintgens T. and Melin T. (2006). Wastewater reuse in Europe. *Desalination*, **187**, 89-101.

Buisson H., Cote P., Praderie M. and Paillard H. (1998). The use of immersed membranes for upgrading wastewater treatment plants. *Water Science and Technology*, **37** (9), 89-95.

Cho B. D. and Fane A.G. (2002). Fouling transients in nominally sub-critical flux operation of a membrane bioreactor. *Journal of Membrane Science,* **209** (2), 391-403.

Cicek N., Winnen H., Suidan M.T., Wrenn B.E., Urbain V. and Manem J. (1998). Effectiveness of the membrane bioreactor in the biodegraddation of high molecular weight compounds. *Water Research,* **32**, 1553-1563.

Côté P., Mourato D., Güngerich C., Russell and Houghton E. (1998). Inmersed membrane filtration for the production of drinking water: Cases studies. *Desalination*, **117**, 181-188.

Countil Directive of 8 December 1975 (1975). Concerning the Quality of Bathing Water, Official. J. Eur. Commun., L31.

Countil Directive of 21 May 1991 (1991). Concerning Urban Waste Water Treatment, Official J.Eur. Commun., L135.

Decree of Environmental Ministry 185/2003. Italian Legislation. Legge quadro sulle acque D. Legs 152/99; Decreto nº 185 12/6/2003.

EPA. (1999). Wastewater technology fact sheet sequencing batch reactor. Office of Water, United States Environmental Protection Agency, Washington DC.

Ficara E., Rocco, A. And Rozzi A. (2000). Determination of nitrification kinetics by the ANITA-DOstat biosensor. *Water Science Technology,* **41** (12), 121-128.

Field R.W., Wu D., Howell J.A. and Gupta B.B. (1995). Critical flux concept for microfiltration fouling. *Journal of Membrane Science*, **100**, 259-272.

Gander M., Jefferson B. and Judd S. (2000). Aerobic MBRs for domestic wastewater treatment: a review with cost considerations. *Separation and Purification Technology,* **18**, 119-130.

Günder B. and Krauth K. (1999). Repplacement of secondary clarification by membrane separation- results with tubular, plante and hollow fibre modules. *Water Science and Technology*, **40** (4-5), 311-320.

Hochstrat R. and Wintgens T. (2003). Draft of wastewater reuse potential estimation, Interim report. Eds. AQUAREC, report on Milestone M3.1.

Howell J.A. (1995). Sub-critical flux operation of microfiltration. *Journal of Membrane Science*, **107**, 165-171.

Irvine R.L., Wilderer P.A. and Flemming H.C. (1997). Controlled unsteady state processes and technologies-an overview. *Water Science and Technology*, **35**, 1-10.

Isra-Cnr (1994). "Metodi analitici per le Acque".

Judd S.J. (2004). A review of fouling of membrane bioreactor in sewage treatment. *Water Science and Technology*, **49** (2), 229-235.

Kang I.J., Lee C.H. and Kim K.J. (2003). Characteristics of microfiltration membranes in a membrane coupled sequencing batch reactor system. *Water Research*, **37**, 1192-1197.

Ketchum Jr. LH. (1997). Design and physical features of sequencing batch reactors. *Water Science and Technology*, **35** (1), 11-8.

Kishino H., Ishida H., Iwabu H. and Nakano I. (1996). Domestic wastewater reuse using a submerged membrane bioreactor. *Desalination*, **106,** 115-119.

Krampe J. and Krauth K. (2000). Sequencing batch reactor with submerged hollow fibre membranes for the biomass separation. *In: Proceedings of 2nd international symposium on sequencing batch reactor technology*, **2**, 109-15.

Kwon D.Y., Vigneswaran S., Fane A.G., Ben Aim R. (2000) Exprimental determination of critical flux in cross-flow microfiltration. *Separation and Purification*, **19**, 169-181.

Larsen T. and Harremoes P. (1994). Degradation mechanisms of colloidal organic matter in biofilm reactors. *Water Research*, **28** (6), 1443-1452.

Melin T., Jefferson B., Bixio D., Thoeye C., De Wilde W., De koning J., van der Graaf J. And Wontgens T. (2006). Membrane bioreactor technology for wastewater treatment and reuse. *Desalination*, **178**, 271-282.

Ognier S., Wisniewski C. and Grasmick A. (2004). Membrane bioreactor fouling in sub-critical filtration conditions: a local critical flux concenpt. *Journal of Membrane Science*, **229** (1-2), 171-177.

Pavelj N., Hvala N., Kocijan J., Ro M., Ubelj M., Mui G. and Strmnik S. (2001). Experimental design of an optimal phase duration control strategy used in batch biological wastewater treatment. *ISA Transactions,* **40** (1), 41-56.

Pochana K. and Keller J. (1999). Study of factors affecting simultaneous nitrification and denitrification (SND*). Water Science and Technology,* **39** (6), 61-8.

Schleypen P., Michel I. and Siewert H.E. (1997). Sequencing batch reactors with continuous inflow for small communities in rural areas in Bavaria. *Water Science and Technology*, **35** (1), 269-276.

Stephenson T., Judd S.J., Jefferson B. and Brindle K. (2000). Membrane Bioreactors for Wastewater Treatment. *IWA Publishing, London.*

Tyszler D., Zytner R..G., Batsch A., Brügger A., Geissler S., Zhou H., Klee D. and Melin T. (2006). Reduced fouling tendencies of ultrafiltration membranes in wastewater treatment by plasma modification. *Desalination*, **189**, 119-129.

Ueda T., Hata K. and Kikuola Y. (1996). Treatment of domestic sewage from rural settlements by a membrane bioreactor. *Water Science Technology,* **34** (9), 189-196.

Ueda T. and Hata K. (1999). Domestic wastewater treatment by a submerged membrane bioreactor with gravitational filtration. *Water Research,* **33** (12), 2888-2892.

Ueda T. and Horan N.J. (2000). Fate of indigenous bacteriaphage in a membrane bioreactor. *Water Research*, **34**, 2151-2159.

Wei Chunhai, Huang Xia and Wen Xianghua (2006). Pilot study on municipal wastewater treatment by a modified submerged membrane bioreactor. *Water Science and Technology,* **53** (9), 103-110.

Conclusions

The main conclusions of this research were to assay and develop different alternatives for the improvement of the biological treatment stage of wastewater treatment plants. Here these are summarised and presented.

Sequencing Batch Reactors (SBRs) were employed to generate and to study the performance of aerobic, nitrifying and Anammox granules in order to improve the nitrogen removal from wastewater. The influence of different parameters (shear stress, biomass selection by means of the settling rate, the type of substrate, COD, N-load and oxygen concentration) in the formation of aerobic/nitrifying/Anammox granules in SBRs was studied.

The formation of aerobic granules in two SBRs was achieved by using an industrial wastewater coming from a dairy analysis laboratory and synthetic wastewater as influent. Granules with good settling properties were obtained, SVI of 60 mL/g VSS, and ZSV of 20 m/h. This made feasible to operate the system with high exchange volume and thus organic and nitrogen loading rates applied to both systems were high, up to 7 g COD/(L·d) and 0.7 g NH_4^+-N/(L·d).

Nitrogen removal efficiency was similar in both units, even considering that R2 was operated always under aerobic conditions. Nitrogen and COD removal efficiencies were 80 and 70 %, respectively. Nitrate disappeared in both units during the first minutes of the feeding period, although in R2 dissolved oxygen concentration was higher than 3 mg O_2/L. Thus, denitrification might take place in R2 in the inner core of the granules, oxygen being depleted by the outer layers.

It was found that the presence of TSS in the effluent was a result of at least of three causes: the own presence of TSS in the influent; the detachment of small biomass patches from the granules and the growth of small flocs that were washed

out. The presence of TSS in the effluent of the SBRs was strongly affected by either the length of the withdrawal period or by the applied particulated COD to biomass ratio (CODp/VSS) to the systems.

Nitrifying granules are easily obtained by removing the organic carbon source from the feeding to an aerobic granular SBR where heterotrophic organic matter oxidation, denitrification and nitrification processes take place. The COD/N ratio influences the composition of the generated effluent by changing the extension of the different processes occurring in the granule. The higher the COD/N ratio up to 5 the higher the N removal percentages.

The effects of hydrodynamic conditions (shear force and reactor configuration) on aerobic granulation were studied in a Sequencing Batch Reactor with an unusual geometry meaning that the H/D ratio of 2.5. Granules with good settling properties were obtained, SVI of 30-40 mL/g VSS, and ZSV higher than 8 m/h. The formation of stable granules was not possible for OLR under 1 g COD/(L·d) meaning that a minimum gradient concentration is needed to be able to generate granular biomass.

Compared to conventional activated sludge processes, where sedimentation takes place in a separate clarifier, the aerobic granular sludge process requires only a limited footprint (20-30% of that of a conventional plant), with clear cost advantages. Also energy savings can be as high as 30-40% by using a granular reactor.

The effect of operating hydrodynamic conditions on the Anammox process were studied in SBR where complete mixture was achieved by means of mechanical stirring or gas flow. It can be concluded that the Anammox process present a high capacity in order to support the high stress supplied by mechanical stirring or gas upflow velocity.

The Anammox process was successfully carried out at specific input power between 0.003 and 0.09 kW/m^3 (up to 180 rpm) for the mechanically stirred reactor and between 0.003 and 0.057 kW/m^3 (up to 5.29 cm/min) for the gas upflow recirculating reactor. However, a reduction on the stability of the process took place when the stirring speed was increased to 250 rpm and the gas upflow

velocity to 7.4 cm/min, which means a specific input power applied to the system of 0.23 kW/m^3 and 0.075 kW/m^3, respectively.

This reduction on the stability affected mainly the specific Anammox activity of the sludge and the biomass retention in the system. Activity of the Anammox granules was decreased which caused a loss of the system efficiency due to a combination of cellular lysis and granules breakage. The biomass retention worsened due to the breakage of the granules and floatation caused by nitrite accumulation. However, the granules were more compact and the density was increased.

When operated at full-scale the control of the shear stress in SBR systems is required in order to perform the process in stable conditions. Shear forces affect to the activity of the Anammox system only in case of application of high stress. When the aim is the formation of granular biomass further research must be focused on the determination of the minimum needed shear stress. It is important to highlight that the activity, the size of the granules and the stability of the process was recovered when the initial shear stress were restored.

The presented work illustrates that membrane technology has a significant potential to become a key element of wastewater reclamation and reuse schemes world-wide. Membranes for municipal wastewater treatment feature advantages compared to conventional activated sludge plants in terms of effluent quality, reflected in lower values for organics, nutrients and microorganisms.

Results demonstrated that the removal efficiency of bacteria and suspended solids by membrane filtration was 100%, suggesting that the experimented compact system (SBR + membrane filtration) could produce an effluent suitable for reuse in agriculture and could be a suitable technology for rural communities. The membrane process coupled with a SBR not only replaces the sedimentation period in the operation of a SBR but also serves as an advanced treatment unit for coliform bacteria and suspended solids, which cannot be removed completely by conventional processes.

To sum up, the new technologies (aerobic, nitrifying and Anammox granular sludge) have shown to be very effective in nutrient removal and are suitable for

the treatment of industrial and municipal wastewaters. Considerable granules dimensions were obtained and the reactors were operated during several months under stable conditions. The study of shear stress, reactor configuration and other parameters contributed to further improve this aspect.

Finally, the use of membrane technologies allowed to operate at high OLR and NLR and was further obtaining an effluent with excellent quality in terms of organic matter, suspended solids and indicator bacteria, which make feasible direct water reuse.

Conclusiones

Se presentan aquí las conclusiones generales de este trabajo de investigación, en el que se evaluaron y desarrollaron diferentes alternativas para la mejora del tratamiento biológico de las aguas residuales.

Se emplearon reactores SBRs (Sequencing Batch Reactors) para la obtención y el estudio del comportamiento de los gránulos aerobios, nitrificantes y Anammox y así estudiar la eliminación de altas concentraciones de nitrógeno del agua residual. Se ha estudiado la influencia de diferentes parámetros (fuerzas de estrés, selección de la biomasa a través de la velocidad de decantación, tipo de substrato, DQO, carga de nitrógeno y concentración de oxígeno) en la formación de gránulos aerobios, nitrificantes y Anammox.

Se obtuvieron gránulos aerobios en dos SBRs, tanto usando agua sintética o agua industrial procedente de un laboratorio de análisis de productos lácteos como influente. Estos gránulos tenían buenas propiedades de sedimentación, índice volumétrico de lodos de 60 mL/g SSV y velocidad de sedimentación de 20 m/h. Esto hizo posible operar el sistema con alto porcentaje de intercambio y con altas velocidades de carga orgánica (VCO) y nitrogenada (VCN) alcanzándose valores de 7 g DQO/(L·d) y 0,7 g NH_4^+-N/(L·d), repectivamente.

Los porcentajes de eliminación de nitrógeno fueron similares en los dos reactores, incluso considerando que el R2 se operó siempre en condiciones aerobias. La eficacia de eliminación de nitrógeno y de DQO fue de 80 y 70%, respectivamente. El nitrato desapareció en los dos sistemas durante los primeros minutos de la alimentación, a pesar de que en R2 la concentración de oxígeno fue siempre superior a 3 mg O_2/L. Esto fue debido a que desnitrificación tuvo lugar en el interior del gránulo donde el oxígeno no penetró.

Se ha encontrado que la presencia de sólidos en suspensión totales (SST) en el efluente de los reactores podría ser un resultado de al menos tres causas: la propia presencia de SST en el influente, el desprendimiento de pequeñas partículas de biomasa del gránulo y el crecimiento de pequeños flóculos los cuales se lavaban del sistema. La presencia de SST en el efluente de los SBRs estuvo relacionada tanto con la longitud de los períodos de vaciado como con la relación DQO particulada/sólidos en suspensión volátiles (DQO/SSV) aplicada al sistema.

Se obtuvieron gránulos nitrificantes fácilmente partiendo de los gránulos heterótrofos en los que se producían los procesos de nitrificación y desnitrificación eliminando la fuente de carbono de la alimentación. Se ha encontrado que la relación DQO/N del influente afecta a la composición del efluente, ya que con el cambio de DQO/N también cambian los procesos que se llevan a cabo en el interior del gránulo. Relaciones DQO/N mayores a 5 producían los mayores porcentajes de eliminación de nitrógeno.

Se ha estudiado también la influencia de las condiciones hidrodinámicas (fuerzas de estrés y configuración del reactor) sobre el proceso de granulación en un SBR con una inusual geometría de altura/diámetro (H/D) de 2,5. Se han obtenido gránulos con buenas propiedades de sedimentación, índice volumétrico de 30-40 mL/g SSV y velocidad de sedimentación mayor de 8 m/h. La formación de gránulos estables no fue posible para VCO menores de 1 g DQO/L·d, lo que implica que se necesita una mínima cantidad de materia orgánica para generar biomasa granular.

Comparado con el proceso de lodos activos, en donde la sedimentación tiene lugar en un decantador separado del reactor, el lodo granular requiere solo 20-30% de espacio en relación a las plantas convencionales, lo cual implica una reducción de costes. También serán menores los requerimientos energéticos, se podrá ahorrar en torno a un 30-40% usando un sistema granular.

Se han estudiado también el efecto de las condiciones hidrodinámicas de operación sobre el proceso Anammox en un SBR, en donde la mezcla completa se alcanzó por medio de un agitador mecánico o por flujo de gas. A la vista de los resultados obtenidos, se puede concluir que el proceso Anammox presenta una

alta capacidad para soportar alto estrés suministrado por la agitación mecánica o por el flujo de gas.

El proceso Anammox se llevó a cabo correctamente trabajando con una potencia específica comprendida entre 0,003 y 0,09 kW/m^3 (hasta 180 rpm) para el reactor agitado mecánicamente y de 0,003 y 0,057 kW/m^3 (hasta 5.29 cm/min) para el reactor agitado por gas. Sin embargo, cuando la velocidad de agitación se incrementó a 250 rpm y el flujo de gas a 7.4 cm/min, lo que implica 0,23 kW/m^3 y 0,075 kW/m^3, respectivamente se produjo una reducción de la estabilidad del proceso.

Esta reducción de la estabilidad afectó principalmente a la actividad específica Anammox y a la retención de la biomasa en el sistema. La actividad Anammox decreció considerablemente, lo que provocó una perdida de eficiencia del sistema debido a la combinación de lisis celular y rotura de gránulos. La retención de biomasa empeoró como consecuencia de la rotura de los gránulos y flotación que estuvo causada por una acumulación de nitrito. Sin embargo, durante este período los gránulos fueron más compactos y la densidad de los mismos también aumentó.

Para la operación a escala industrial de un SBR se deberá realizar un control de las fuerzas de estrés para que el proceso Anammox se lleve a cabo de forma estable. Estas fuerzas afectan a la actividad del sistema Anammox sólo en el caso de aplicar un alto estrés. Es importante también subrayar que la actividad, el tamaño de los gránulos y la estabilidad del proceso se recuperó una vez que se recuperó el estrés inicial aplicado al sistema.

El presente trabajo demostró que la tecnología de membranas tiene una gran importancia para llegar a ser un elemento clave para el tratamiento y reutilización de aguas. Las membranas presentan grandes ventajas para el tratamiento de aguas residuales urbanas comparadas con los sistemas tradicionales de depuración de aguas. Entre estas ventajas señalar, la alta calidad del efluente reflejada en valores muy bajos de compuestos orgánicos, nutrientes y microorganismos.

Los resultados obtenidos demostraron que la eficacia de eliminación de bacterias y sólidos en suspensión en sistemas de membranas fue de un 100%, lo cual indica que el sistema compacto (SBR + membrana) puede producir un

efluente apto para su reutilización en agricultura y podría ser una tecnología apropiada para comunidades rurales. El sistema de membrana acoplado a un SBR no sólo reemplaza el período de sedimentación sino que también sirve como una unidad de tratamiento avanzado para la eliminación de bacterias coliformes y sólidos en suspensión, los cuales no se pueden eliminar totalmente con los sistemas de tratamiento convencionales.

Resumiendo, las nuevas tecnologías (gránulos aerobios, nitrificantes y Anammox) han mostrado ser muy eficientes en la eliminación de nutrientes y son apropiadas para el tratamiento tanto de aguas municipales como industriales. Gránulos de un tamaño considerable se han obtenido en los diferentes reactores y se han operado de forma estable durante varios meses. El estudio de las fuerzas de estrés, la configuración del reactor y otros parámetros contribuyeron a mejorar la estabilidad del proceso.

Por último, el uso de la tecnología de membranas permitió obtener un efluente con alta calidad en términos de materia orgánica, sólidos en suspensión y bacterias coliformes, lo cual permite la reutilización directa de las aguas residuales.

Conclusións

Preséntanse aquí as conclusións xerais deste traballo de investigación, no que se avaliaron e desenrolaron diferentes alternativas para a mellora do tratamento biolóxico das augas residuais.

Empregáronse reactores SBRs (Sequencing Batch Reactors) para a obtención e o estudio do comportamento dos gránulos aerobios, nitrificantes e Anammox e así estudiar a eliminación de altas concentracións de nitróxeno da auga residual. Estudiouse a influencia de diferentes parámetros (forzas de estres, selección da biomasa a través da velocidade de decantación, tipo de substrato, DQO, carga de nitróxeno e concentración de osíxeno) na formación de gránulos aerobios, nitrificantes e Anammox.

Obtivéronse gránulos aerobios en dous SBRs, tanto usando como influente auga sintética como auga industrial procedente dun laboratorio de análise de productos lácteos. Estes gránulos tiñan boas propiedades de sedimentación, índice volumétrico de lodos de 60 mL/g SSV e velocidade de sedimentación de 20 m/h. Esto fixo posible operar o sistema con alto porcentaxe de intercambio e con altas velocidades de carga orgánica (VCO) e nitroxenada (VCN) alcanzándose valores de 7 g DQO/(L·d) e 0,7 g NH_4^+-N/(L·d), respectivamente.

Os porcentaxes de eliminación de nitróxeno foron similares nos dous reactores, incluso considerando que o R2 operouse sempre en condicións aerobias. A eficacia de eliminación de nitróxeno e de DQO foi de 80 e 70%, respectivamente. O nitrato desapareceu nos dous sistemas durante os primeiros minutos da alimentación, a pesar de que en R2 a concentración de osíxeno foi sempre superior a 3 mg O_2/L. Esto foi debido a que a desnitrificación tivo lugar no interior do gránulo donde o osíxeno non penetrou.

Atopouse que a presencia de sólidos en suspensión totais (SST) no efluente dos reactores podía ser un resultado de polo menos tres causas: a propia presencia de SST no influente, o desprendemento de pequenas partículas de biomasa do gránulo e o crecemento de pequenos flóculos os cales lavábanse do sistema. A presencia de SST no efluente dos SBRs estivo relacionada tanto ca lonxitude dos períodos de vertido como ca relación DQO particulada/sólidos en suspensión volátiles (DQO/SSV) aplicada ó sistema.

Obtivéronse gránulos nitrificantes facilmente partindo dos gránulos heterótrofos nos que se producían os procesos de nitrificación e desnitrificación eliminando a fonte de carbono da alimentación. Encontrouse que a relación DQO/N influenciou a composición do efluente, xa que co cambio de DQO/N tamén cambiaban os procesos que se levan a cabo no interior do gránulo. Relacións DQO/N maiores a 5 producían os maiores porcentaxes de eliminación de nitróxeno.

Estudiouse tamén a influencia das condicións hidrodinámicas (forzas de estres e configuración do reactor) sobre o proceso de granulación nun SBR con unha xeometría de altura/diámetro (H/D) de 2,5. Obtivéronse gránulos con boas propiedades de sedimentación, índice volumétrico de 30-40 mL/g SSV, e velocidade de sedimentación maior de 8 m/h. A formación de gránulos estables non foi posible para VCO menores de 1 g DQO/L·d, o que implica que se necesita unha mínima cantidade de materia orgánica para xerar biomasa granular.

Comparado co proceso de lodos activos, en donde a sedimentación ten lugar nun decantador separado do reactor, o lodo granular requiro só un 20-30% do espacio en relación as plantas convencionais, o cal implica unha reducción de costes. Tamén serán menores os requirimentos enerxéticos, poderase aforrar en torno a un 30-40% usando un sistema granular.

Estudiouse tamén o efecto das condicións hidrodinámicas de operación sobre o proceso Anammox en un SBR, en donde a mixtura completa alcanzouse usando un axitador mecánico ou por fluxo de gas. Á vista dos resultados obtidos, pódese concluír que o proceso Anammox presenta unha alta capacidade para soportar alto estres subministrado pola axitación mecánica ou polo fluxo de gas.

O proceso Anammox levouse a cabo correctamente traballando cunha potencia específica comprendida entre 0,003 e 0,09 kW/m^3 (ata 180 rpm) para o reactor axitado mecanicamente e de 0,003 e 0,057 kW/m^3 (a 5.29 cm/min) para o reactor axitado por gas. Sen embargo, cando a velocidade de axitación incrementouse a 250 rpm e o fluxo de gas a 7,4 cm/min, o que implicou 0,23 kW/m^3 e 0,075 kW/m^3, respectivamente obtívose unha reducción da estabilidade do proceso.

Esta reducción da estabilidade afectou principalmente á actividade específica Anammox e á retención da biomasa no sistema. A actividade Anammox diminuíu considerablemente, o que provocou unha perdida de eficiencia do sistema debido a combinación de lise celular e a rotura dos gránulos. A retención da biomasa empeorou como consecuencia da rotura dos gránulos e a flotación que estivo causada pola acumulación de nitrito. Sen embargo, durante este período os gránulos foron máis compactos e a densidade dos mesmos tamén aumentou.

Para a operación a escala industrial dun SBR deberase realizar un control das forzas de estres para que o proceso Anammox lévese a cabo de forma estable. Estas forzas afectan á actividade do sistema Anammox só no caso de aplicar un alto estres. É importante subliñar que a actividade, o tamaño dos gránulos e a estabilidade do proceso recuperouse unha vez que volveu o estres inicial aplicado ó sistema.

O presente traballo demostrou que a tecnoloxía de membranas ten unha gran importancia para chegar a ser un elemento clave para o tratamento e a reutilización de augas. As membranas presentan grandes vantaxes para o tratamento de augas residuais urbanas comparadas cos sistemas tradicionais de depuración de augas. Entre estas vantaxes subliñar, a alta calidade do efluente reflexada en valores moi baixos de compostos nitroxenados, nutrientes e microorganismos.

Os resultados obtidos demostraron que a eficacia de eliminación de bacterias e sólidos en suspensión en sistemas de membranas foi dun 100%, o cal indica que o sistema compacto (SBR + membrana) pode producir un efluente apto para a súa reutilización na agricultura e podería ser unha tecnoloxía apropiada para comunidades rurais. O sistema de membrana acoplado a un SBR non só

reemplaza o período de sedimentación senón que tamén serve como unha unidade de tratamento avanzado para a eliminación de bacterias coliformes e sólidos en suspensión, os cales non se poden eliminar totalmente cós sistemas de tratamento convencionais.

Resumindo, as novas tecnoloxías (gránulos aerobios, nitrificantes e Anammox) mostraron ser moi eficientes na eliminación de nutrientes e son apropiadas para o tratamento tanto de augas municipais como industriais. Obtivéronse gránulos dun tamaño considerable nos diferentes reactores e operáronse de forma estable durante varios meses. O estudio das forzas de estres, a configuración do reactor e outros parámetros contribuíron a mellorar a estabilidade do proceso.

Por último, o uso da tecnoloxía de membranas permitiu obter un efluente con alta calidade en termos de materia orgánica, sólidos en suspensión e bacterias coliformes, o cal permite a reutilización directa das augas residuais tratadas.

List of publications

1. Journal papers

- **Arrojo B.,** Figueroa M., Mosquera-Corral A., Campos J.L. and Méndez R. (2006). Effects of hydrodynamic conditions and feeding composition on the aerobic granulation in SBRs. *(Submitted)*.
- **Arrojo B.,** Vázquez-Padín J.R., Figueroa M., Mosquera-Corral A., Campos J.L. and Méndez R. (2006). Reactores de biomasa granular: Más capacidad en menos espacio. *Afinidad. (Submitted).*
- Vázquez-Padín J.R., Figueroa M., **Arrojo B**., Mosquera-Corral A., Campos J.L. and Méndez R. (2006) Why do nitrifying granules accumulate nitrite?. *(Submitted).*
- **Arrojo B.,** Figueroa M., Mosquera-Corral A., Campos J.L. and Méndez R. (2006). Effects of hydrodynamic shear forces on Anammox granules. (*Submitted*).
- Campos J.L., **Arrojo B.,** Mosquera-Corral A. and Méndez R. (2006). N_2O production by nitrifying biomass under aerobic and anoxic conditions. *Applied Biochemistry and Biotechnology*. (*Submitted*).
- **Arrojo B**., Mosquera-Corral A., Campos J.L. and Méndez R. (2006). Effects of mechanical stress on Anammox granules in a sequencing batch reactor (SBR). *Journal of Biotechnology*, **123**, 453-563.
- **Arrojo B.**, Mosquera-Corral A., Garrido J.M., Méndez R., Ficara E. and Malpei F (2005). Membrane coupled to a Sequencing Batch Reactor for water reuse and removal of coliform bacteria. *Desalination,* **179**, 109-116.
- **Arrojo B.**, Mosquera-Corral A., Garrido J.M. and Méndez R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. . *Water Research,* **38**, 3389-3399.
- Dapena-Mora A., **Arrojo B.**, Campos J.L., Mosquera-Corral A. and Méndez R. (2004). Improvement of the settling properties of Anammox sludge in a SBR. *Journal of Chemical Technology and Biotechnology*, **79**, 1417-1420.
- Fernández-Carrasco E. Omil F., Garrido J.M., **Arrojo B.** and Méndez R. (2004). Advanced monitoring and supervision of biological treatment of complex dairy effluents in a full-scale plant. *Biotechnology Progress*, **20,** 992-997.
- **Arrojo B.**, Omil F., Garrido J.M. and Méndez R. (2003). Combinación de un filtro anaerobio y un sistema SBR para el tratamiento de las aguas generadas en un laboratorio de análisis de productos lácteos. *Afinidad*, **60**, 344-354.
- Omil F., Garrido J.M., **Arrojo B**., and Méndez R. (2003). Anaerobic filter reactor performance during the treatment of complex dairy wastewaters at industrial scale. *Water Research***, 37**, 4099-4108.
- Garrido J.M., Omil F., **Arrojo B.**, Méndez R. and Lema J.M. (2001). Carbon and nitrogen renoval from a wastewater of an industrial dairy laboratory with a coupled anaerobic filter-sequencing batch reactor system. *Water Science and Techno*logy, **43**, 249-256.

2. Book chapters

- Mosquera-Corral A., Vázquez –Padín J.R., **Arrojo B**., Campos J.L. and Méndez R. (2005). Nitrifying granular sludge in a Sequencing Bacth Reactor. *Aerobic Granular Sludge. Water and environmental Management series, IWA Publishing*, 63-70.

3. Contribution to congress

- Vázquez-Padín J.R., Figueroa M., **Arrojo B**., Mosquera-Corral A., Campos J.L. and Méndez R.. Why do nitrifying granules accumulate nitrite?. Oral presentation. *2nd Aerobic granular sludge Workshop*. Delf, The Netherlands. September 2006.

- Vázquez-Padín J.R., Figueroa M., **Arrojo B**., Campos J.L., Mosquera-Corral A. and Méndez R. Tratamiento de efluentes industriales en sistemas de granulación aerobia. Poster. Biospain- BIOTEC 2006. Madrid, Spain. September 2006.

- **Arrojo B**., Mosquera-Corral A., Campos J.L. and Méndez R. Effects of hydrodynamic shear force on Anammox granules. Oral presentation. *International Symposium on Environmental Biotechnology ESEB 2006.* Leipzig, Germany. July 2006

- **Arrojo B**., Mosquera-Corral A., Campos J.L. and Méndez R. How does stress affect Anammox granulation and performance?. Oral presentation. *International Water Conference (IWC'2006*). Porto, Portugal. June 2006.

- Dapena-Mora A., Trigo C., Fernández I., Vázquez-Padín J.R., Figueroa M., **Arrojo B**., Garrido J.M., Mosquera-Corral A., Campos J.L. and Méndez R. Start-up of Anammox reactors: Different reactor alternatives. Oral presentation. *IWA Specialized Conference Nutrient Management in Wastewater Treatment Processes and Recycle Streams*. Krakow, Poland. September 2005.

- Vázquez-Padín J.R., **Arrojo B**., Mosquera-Corral A., Figueroa M., Campos J.L. and Méndez R. Granulation sludge for aerobic wastewater treatment: nitrogen renoval. Poster. *X Congreso Mediterráneo de Ingeniería Química*. Barcelona, Spain. November 2005.

- **Arrojo B.**, Mosquera-corral A., Garrido J.M., Méndez R, Ficara E. and Malpei F. Membrane coupled to a sequencing batch reactor for water resuse and removal of coliform bacteria. Oral presentation. *Congress on Membranes in Drinking and Industrial Water Production*. L'Aquila, Italy. November 2004.

- Mosquera-Corral A., Vázquez –Padín J.R., **Arrojo B**., Campos J.L. and Méndez R. Nitrifying granular sludge in a Sequencing Bacth Reactor. Oral presentation. *IWA workshop on Aerobic Granular Sludge*. Munich (Germany). September 2004.

- **Arrojo B.**, Mosquera-Corral A., Campos J.L., Garrido J.M. and Méndez R. Eliminación de nitrógeno en un reactor continuo secuencial granular (SBR). Poster. *Congreso Nacional de Biotecnología* (BIOTEC'2004). Oviedo, Spain. July 2004.
- **Arrojo B.**, Mosquera-Corral A., Campos J.L. and Méndez R. Nitrite production in a granular Sequencing Batch Reactor. Poster. *European Symposium on Environmental Biotechnology ESEB 2004*. Oostende, Belgium. April 2004.
- **Arrojo B.**, Mosquera-Corral A., Garrido J.M. and Méndez R.. Aerobic granulation in a SBR fed with industrial wastewater. Poster. *European Symposium on Environmental Biotechnology ESEB 2004*. Oostende, Belgium. April 2004.
- **Arrojo B.**, Mosquera-Corral A., Campos J.L., Garrido J.M. and Méndez R.. Influence of COD/N ratio on N removal in aerobic granular sludge. Poster. *EU 5 th Framework ICON-Anammox 2004 symposium "New sustainable N-removal from wastewater*". Ghent , Belgium. January 2004.
- **Arrojo B.**, Mosquera-Corral A., Garrido J.M. and Méndez R. Aerobic granulation in SBR with industrial wastewater. Poster. *4th European Congress of Chemical Engineering*. Granada, Spain. September 2003.
- **Arrojo B.**, Garrido J.M., Omil F., and Méndez R. Treatment of dairy wastewaters at industrial scale with a coupled anaerobic filter-sequencing batch reactor. Poster. *9th Mediterranean Congress of Chemical Engineering*. Barcelona, Spain. November 2002.
- Omil F., Garrido J.M., **Arrojo B**., Pena R., Méndez R. Anaerobic treatment of the effluents discharged by milk analysis laboratories. Poster. *9 th World Congress Anaerobic Digestion 2001*. Antwerpen, Belgium. September 2001.
- Garrido J.M., Omil F., **Arrojo B.**, Méndez R., Lema J.M. Carbon and nitrogen removal from a wastewater of an industrial dairy laboratory with a coupled anaerobic filter-sequencing batch reactor system. Oral presentation. *2nd International Symposium on Sequencing Batch Reactor Technology*. Narbonne, France. July 2000.
- S. López, **Arrojo B.**, M. Jull and J.M. Garrido. Treatment of a dairy wastewater in an anaerobic filter. Poster. *IV International ANQUE Chemistry Conference Food*. Lugo, Spain. September 1998.
- **Arrojo B.**, S. López, E. Roca and J.M. Garrido. Treatment of the water of a laboratory for milk analysis in an anaerobic filter. Poster. *IV Iberian Congress on Biotechnology*, BIOTEC´98. Guimaraes, Portugal. July 1998.