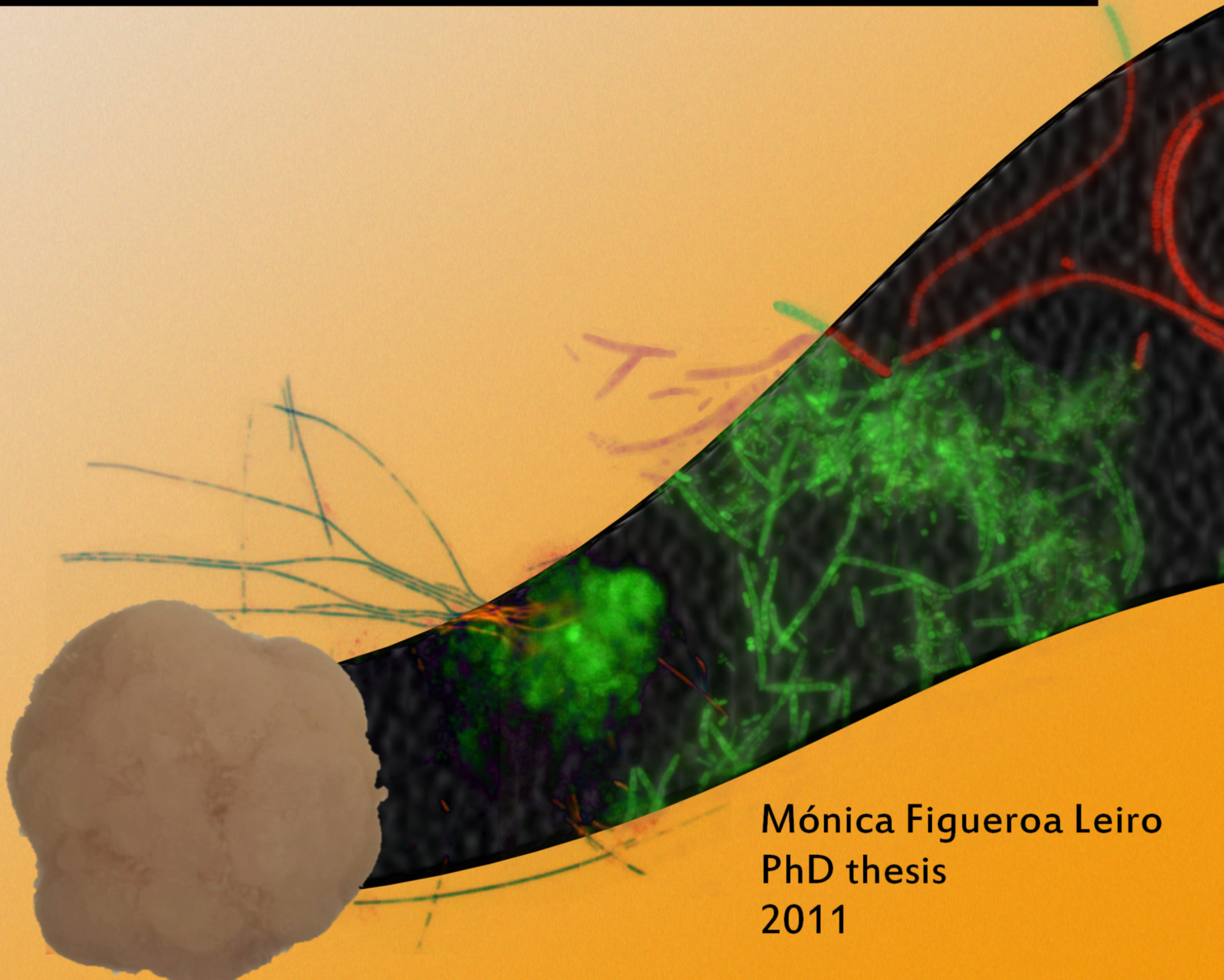
Aerobic granular systems for biological treatment of industrial wastewater:

Operation and characterization of microbial populations



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Informan:

Que la memoria titulada "Aerobic granular systems for biological treatment of industrial wastewater: operation and characterization of microbial populations", que para optar al grado de Doctor de Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Doña Mónica Figueroa Leiro, ha sido realizada 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 9 de abril de 2011.

Ramón Méndez Pampín   Anuska Mosquera Corral

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"Lo que dejamos atrás y lo que tenemos por delante no son nada comparado con lo que llevamos dentro." (Ralph Waldo Emerson)

“What lies behind us and what lies before us are small matters compared to what lies within us.” (Ralph Waldo Emerson)

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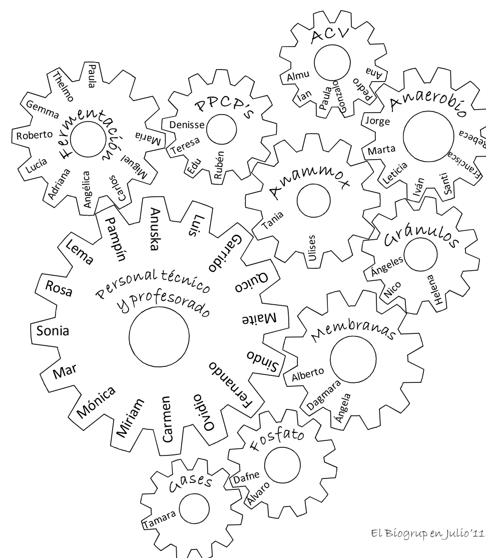

El Biogrup en Julio'11

El Biogrup está compuesto por múltiples engranajes: ACV, digestión anaerobia, fermentación, membranas, PPCP's… y cada uno

de los integrantes de estos equipos aportó su grano de arena para que todo saliese bien y fuese más llevadero durante todos estos años: un truco con un equipo, una mano con el reactor, los análisis de muestras, unas garrafas de agua, el papeleo, una sonrisa por los pasillos, el café y unos pasteles a media mañana o esas sobremesas en las que cada día surge un nuevo tema de conversación… El personal técnico del Biogrup se merece un reconocimiento especial: por cada muestra analizada, por cada proyecto presentado y por cada pago tramitado. Sois los testigos de nuestro paso por el Biogrup: de cómo entramos con cara de desconcierto por la puerta y de cómo vamos aprendiendo a desenvolvernos.

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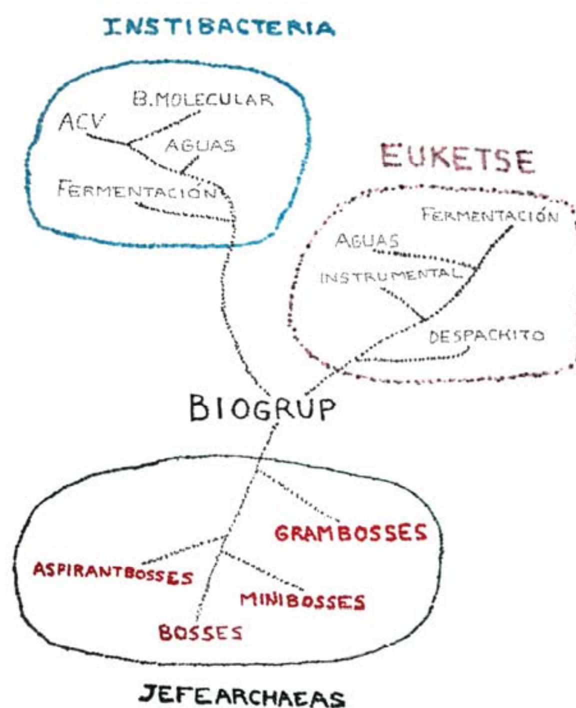

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Resumen

Las nuevas normativas y políticas, tanto a nivel estatal como comunitario, son cada vez más exigentes en cuanto a las concentraciones de contaminantes en los efluentes generados tanto a nivel industrial como urbano. Este hecho, junto con la reducción de las superficies disponibles para su gestión, está impulsando el desarrollo de nuevos sistemas y tecnologías para el tratamiento de las aguas residuales más eficaces y compactos en los cuales se pueda llevar a cabo la eliminación conjunta de materia orgánica y nutrientes, como el nitrógeno y el fósforo.

El empleo de reactores secuenciales (SBR: Sequencing Batch Reactors) con biomasa granular aerobia aplicados al tratamiento biológico de aguas residuales en condiciones aerobias se presenta como una alternativa al proceso convencional de lodos activos. Estos reactores se operan en forma secuencial en ciclos que comprenden fases de llenado, reacción, sedimentación y vaciado. Los ciclos de operación se caracterizan por la corta duración de las fases de llenado y de sedimentación con el fin de desarrollar la biomasa en forma de gránulos en condiciones aerobias.

Estos sistemas permiten tratar cargas más elevadas que los sistemas de lodos activos debido a la acumulación de grandes concentraciones de biomasa. Por otro lado, las buenas características de sedimentabilidad que presentan los gránulos aerobios y la baja productividad de biomasa permiten prescindir de los grandes y costosos sedimentadores secundarios, requiriéndose por tanto menos espacio para su implantación. Otra de las ventajas de estos sistemas granulares es que en ellos se pueden llevar a cabo simultáneamente los procesos de eliminación de la materia orgánica y de nitrógeno por medio de los procesos de nitrificación y desnitrificación simultánea (SND). De esta forma se logra no sólo la mejora de la calidad de los efluentes generados sino también una reducción de los costes de implementación y de gestión de lodos.

Los primeros estudios centrados en la granulación de biomasa aerobia se desarrollaron en reactores a escala laboratorio utilizando medios sintéticos para poder comprender mejor todas las variables que afectaban al proceso de formación. De esta forma se determinó que entre los factores clave de la operación de estos sistemas se encuentran el tiempo de decantación, las de estrés creadas por las condiciones hidrodinámicas o mecánicas, el tipo de sustrato, la concentración de materia orgánica (DQO) y de nitrógeno, la concentración de oxígeno en el medio líquido o la presencia de posibles sustancias inhibitorias.

El conocimiento de las condiciones óptimas de operación para la formación de los gránulos ha permitido reducir el tiempo de puesta en marcha de estos sistemas. A partir de aquí, las líneas de investigación en este campo se han diversificado con el paso de los años hacia temas más específicos que van desde el estudio del efecto de la hidrodinámica de los reactores, las limitaciones en cuanto a la transferencia de masa o el estudio de las características de los gránulos tras periodos largos de almacenamiento. En la mayoría de los casos los trabajos se han realizado utilizando un medio sintético. Con el fin de evaluar la aplicabilidad de esta tecnología para tratar efluentes generados en distintos sectores industriales se han realizado estudios tratando aguas residuales, en reactores a escala laboratorio y más tarde a escala planta piloto. Esta información es imprescindible para poder realizar el escalado del sistema a nivel industrial, puesto que ésta es la finalidad última del diseño de las nuevas tecnologías.

Usando como base los conocimientos existentes (**Capítulo 1**), en la presente tesis se ha estudiado la puesta en marcha y operación de reactores granulares aerobios empleando dos tipos diferentes de agua residual con el fin de estudiar la eliminación de materia orgánica y nitrógeno junto con las propiedades físicas de los gránulos generados. Los efluentes estudiados se obtuvieron de una industria conservera de productos marinos (**Capítulo 3**) y de una granja de producción de ganado porcino (**Capítulos 5** y **6**) puesto que son dos efluentes generados a raíz de actividades económicas de gran importancia en Galicia. Las características comunes de estas dos corrientes son el gran volumen de agua residual a tratar y las elevadas concentraciones de materia orgánica y nitrógeno que contienen.

Cuando se trabaja con sistemas biológicos es muy importante considerarlos no sólo como cajas negras sino prestar atención a los organismos implicados en los diferentes procesos. Las técnicas de biología molecular como la DGGE (Denaturing Gradient Gel Electrophoresis), construcción de librerías de clones y FISH (Fluorescent *in situ* Hibrydization) permiten identificar y cuantificar a los microorganismos, así como seguir su dinámica poblacional a lo largo de la operación de los sistemas biológicos. Estas técnicas de biología molecular se utilizaron para identificar las principales poblaciones microbianas que llevaban a cabo los procesos biológicos de los reactores utilizados (**Capítulos 4, 7 y 8**).

Los contenidos principales de cada uno de los capítulos de la presente tesis y los objetivos alcanzados son los siguientes:

En el **Capítulo 1** se presenta una revisión bibliográfica de los diferentes actores implicados en la depuración de aguas residuales. Por una parte se indican los motivos conducentes a la necesidad del establecimiento de sistemas de tratamiento. A continuación se hace una breve descripción de los procesos biológicos de eliminación de materia orgánica y nitrógeno en las aguas residuales incluyendo aspectos como la estequiometría, parámetros cinéticos o el efecto inhibitorio de determinados compuestos. Se presenta también información relativa a los sistemas de granulación aerobia y su estado del arte, incluyendo los

estudios realizados hasta la fecha tratando agua residual de origen industrial o urbano a escala laboratorio y los llevados a cabo en instalaciones a escala planta piloto. Finalmente, se revisan las características de las principales poblaciones bacterianas implicadas en los procesos biológicos de eliminación de nitrógeno (bacterias oxidantes de amonio, bacterias oxidantes de nitrito, bacterias anammox y bacterias desnitrificantes) haciendo una breve descripción de los aspectos más relevantes relacionados con su actividad.

En el **Capítulo 2** se desarrollan los materiales y métodos utilizados en los experimentos realizados en los capítulos posteriores. Esto incluye, tanto los parámetros convencionales de caracterización de la fase líquida de las aguas residuales (materia orgánica, compuestos nitrogenados, pH, oxígeno disuelto y compuestos de carbono), como la caracterización de la biomasa granular desde el punto de vista de sus propiedades físicas y actividades biológicas hasta el de las poblaciones microbianas presentes en la misma. Por un lado se determinaron parámetros como la densidad de los gránulos, el índice volumétrico de lodos y se utilizaron técnicas basadas la observación con estereomicroscopio para la estimación del diámetro medio y la distribución de tamaños de partícula. Por otro lado se detallan las técnicas de biología molecular utilizadas para la identificación de las distintas poblaciones bacterianas y que se basan en la amplificación de ADN, aislamiento y secuenciación. Se presentan los distintos cebadores utilizados para la amplificación del gen 16S del ARN ribosómico y del gen amoA, que codifica la enzima amonio monooxigenasa, el modo en que se ha utilizado la técnica DGGE y el procedimiento para la construcción de librería de clones para obtener la separación de los distintos fragmentos de ADN. Además se describe el protocolo utilizado para aplicar la técnica FISH junto con una clasificación detallada de todas las sondas que se han aplicado a lo largo de los distintos capítulos de esta tesis. Se incluye asimismo en el presente capítulo el procedimiento para el cálculo de ciertos parámetros con el fin de evaluar la operación de los reactores utilizados.

En el **Capítulo 3** se estudia la puesta en marcha y operación de un reactor granular aerobio para tratar agua residual procedente de una industria conservera de productos marinos. En la Comunidad de Galicia estas industrias, especialmente las ubicadas en zonas costeras, se suelen caracterizar por la poca disponibilidad de espacio para la instalación de un sistema de tratamiento convencional de aguas residuales, la variabilidad en la composición y la presencia de sustancias potencialmente inhibitorias como cloruros en el efluente producido. Por sus ventajas, el uso de sistemas granulares sería una alternativa viable para el tratamiento de estos efluentes. En este capítulo se utilizó un reactor SBR con biomasa granular en condiciones aerobias a escala laboratorio (1,5 L) para tratar el efluente de una planta de enlatado de conservas de pescado localizada en la ría de Vigo. Esta corriente presentaba un contenido elevado de cloruros procedentes del uso de agua de mar en el proceso industrial susceptibles de afectar a la actividad biológica por lo que la puesta en marcha del sistema se realizó aplicando diferentes diluciones con agua. Se observó que aunque el proceso de granulación fue más lento que en los sistemas que utilizaban un medio

sintético, al cabo de 75 días de operación, los gránulos eran estables. En este momento los gránulos obtenidos presentaban un diámetro de 3,2 mm, un índice volumétrico de lodos de 30 mL/g SST y una concentración de sólidos de 12 g SSV/L. El factor de dilución aplicado a la alimentación se fue disminuyendo progresivamente y el sistema pudo tratar de forma simultánea una velocidad de carga orgánica de 1,46 kg DQO/(m^3 d) y una velocidad de carga nitrogenada de 0,18 kg N/(m^3 d) con eficacias de eliminación del 95% y 40%, respectivamente. Se evaluó también la evolución de los perfiles de concentración de materia orgánica y nitrógeno a lo largo de ciclos de operación correspondientes a las distintas condiciones operacionales. Finalmente, mediante ensayos de respirometría, se comprobó como la actividad nitrificante de los gránulos está íntimamente relacionada con su diámetro. Así, a mayor diámetro la actividad nitrificante es menor debido a mayores limitaciones en la transferencia de masa y el desarrollo de poblaciones heterótrofas que se desarrollan a mayor velocidad y que compiten por el mismo substrato.

En el **Capítulo 4** se realiza la caracterización y el seguimiento de las principales poblaciones bacterianas presentes en la biomasa del reactor granular mediante el uso de técnicas moleculares. La técnica DGGE mostró la evolución de las poblaciones microbianas desde la puesta en marcha hasta el final de la operación del reactor evaluadas de acuerdo a la presencia o ausencia de bandas características. La secuenciación de las bandas más representativas permitió identificar a los géneros *Bacteroidetes* y *Thauera* como los representativos de las primeras etapas de operación, y *Thiothrix* y *Chloroflexi* como los representativos de las últimas etapas. La construcción de la librería de clones permitió identificar las principales bacterias oxidantes de amonio como pertenecientes al género *Nitrosomonas* y también otros miembros de la población heterótrofa como pertenecientes a la familia *Comamonadaceae* y distintos géneros de la familia *Rhodocyclaceae*. En los gránulos aerobios analizados se identificaron dos géneros diferentes de bacterias filamentosas, *Chloroflexi* y *Thiothrix*, que podrían actuar como un esqueleto en el proceso de formación del gránulo. La aplicación de la técnica FISH sobre microcortes de los gránulos permitió visualizar la distribución espacial de las bacterias oxidantes de amonio y las bacterias del género *Thiothrix*.

En el **Capítulo 5** se estudia la puesta en marcha y operación de un reactor granular para tratar la fracción líquida del purín generado en una granja porcina sita en Santiago de Compostela. Los purines de cerdo se caracterizan por su elevado contenido en materia orgánica y nitrógeno debido al material fecal y la orina de los animales. La aplicación de estos efluentes sobre los terrenos agrícolas como medio fertilizante es una alternativa para su gestión pero no es una solución válida cuando la extensión de terreno disponible es limitada ya que hay que considerar cuestiones como el sobreabonado y que la legislación vigente marca el valor máximo de aplicación en 170 kg N/Ha de terreno, lo que hace necesario por tanto un tratamiento, previo al vertido, de estos purines. En la actualidad se está potenciando la digestión anaerobia como alternativa de valorización, ya que permite el aprovechamiento

energético del biogás producido, pero tiene la desventaja de su baja eficacia en cuanto a la eliminación de nitrógeno. La utilización de sistemas granulares aerobios permitiría la eliminación simultanea de nitrógeno y materia orgánica. Además su diseño compacto y su fácil operación lo harían adecuado para su instalación en las granjas ganaderas. Para evaluar la idoneidad de los sistemas granulares se utilizó un reactor con un volumen de 1,5 L alimentado con la fracción líquida de purín de cerdo diluida con agua. Los primeros gránulos aerobios se observaron al cabo de 15 días de operación y se mantuvieron estables durante todo el proceso. Como ejemplo en el día 140 de operación la concentración de sólidos en el interior del reactor era de 13 g SST/L y los gránulos aerobios tenían un diámetro medio de 5 mm, un índice volumétrico de lodos de 32 mL/g SST y una densidad alrededor de 55 g $SSV/L_{gránulo}$. Se estudió la estabilidad del sistema a medida que se aumentaba la velocidad de carga a tratar. Con valores de carga aplicada de 4,4 kg DQO/(m^3 d) y 0,83 kg N/(m^3 d) el sistema eliminaba simultáneamente el 87% de la materia orgánica y el 70% de nitrógeno. Para poder tratar el purín sin necesidad de dilución previa se efectuó una reducción del volumen de intercambio lo que produjo el crecimiento excesivo de biomasa en suspensión con propiedades de sedimentabilidad peores que la biomasa granular. Sin embargo se comprobó que el sistema seguía eliminando con eficacia similar la materia orgánica, con cargas de hasta 7,0 kg DQO/(m^3 d) mientras que la eliminación de nitrógeno se vio reducida por lo que se hizo necesaria la aplicación de un post-tratamiento para la eliminación de nitrógeno.

En el **Capítulo 6** se estudia la viabilidad de la aplicación del proceso CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) para tratar el efluente del reactor granular aerobio alimentado con purín sin diluir puesto que esa corriente de salida, y de forma análoga la de los sistemas de digestión anaerobia, se caracterizaba por una baja concentración de materia orgánica y un elevado contenido de nitrógeno (baja relación DQO/N). En estos sistemas CANON coexisten dos grupos de bacterias autótrofas, las bacterias oxidantes de amonio y las bacterias anammox, de forma que las primeras oxidan parte del amonio a nitrito y las últimas combinan ambos para producir nitrógeno gas. Aunque el proceso anammox ya ha sido utilizado anteriormente con efluentes de la digestión anaerobia de purines, este trabajo es uno de los primeros en los que se utiliza el proceso CANON para su post-tratamiento y además, operado a baja temperatura. Para este estudio se utilizó reactor con un volumen de 1,5 L al que se suministra el oxígeno mediante pulsos de aire con el fin de mantener la concentración de oxígeno en el medio líquido en los valores adecuados para las bacterias oxidantes de amonio, no afectar a las bacterias anammox y favorecer la mezcla y la transferencia de materia en el líquido. En este caso se evaluó la capacidad del sistema para tratar el efluente producido en el reactor granular aerobio, llegando a una velocidad de carga aplicada de 1,25 kg N/(m^3 d) y a una eficacia de eliminación del 75%. En este capítulo se realiza asimismo un estudio comparativo de la aplicación del sistema CANON con otros procesos que se han planteado en los últimos años para la eliminación de nitrógeno de los efluentes ganaderos.

En el **Capítulo 7** se profundiza en las características físicas y en la caracterización de las poblaciones microbianas de la biomasa presente en el reactor CANON. Se pretende evaluar la coexistencia entre las bacterias heterótrofas, que se podrían desarrollar debido a la presencia de materia orgánica y las condiciones microaerobias utilizadas, y las bacterias autótrofas (anammox y oxidantes de amonio). La biomasa de este reactor se desarrolló distribuida en dos fracciones: una en forma de gránulos con un diámetro medio de 2,6 mm y con un color rojizo intenso, debido a la presencia de bacterias anammox, y otra en suspensión, compuesta principalmente por bacterias oxidantes de amonio y heterótrofas. Mediante la realización de una librería de clones y la aplicación de la técnica de DGGE se identificaron miembros del filo *Bacteroidetes* y de la familia *Comamonadaceae* y del género *Zoogloea* pertenecientes a la población de bacterias heterótrofas. El uso de cebadores específicos para la determinación de la población anammox reveló la presencia de bacterias pertenecientes al género *Candidatus “*Brocadia anammoximans” y *Candidatus* “Brocadia fulgida”. Finalmente, se identificaron las bacterias oxidantes de amonio, que pertenecían al género *Nitrosomonas*. El uso de la técnica FISH permitió realizar una estimación de la abundancia de los principales organismos implicados en el proceso CANON, con una abundancia relativa del 35% de bacterias oxidantes de amonio y 30% de anammox. La presencia de bacterias heterótrofas en sistemas anammox podría ser la responsable de problemas de competición por sustratos comunes como el nitrito para desnitrificar. Sin embargo, gracias a las condiciones de operación del reactor, no se observó la desestabilización del sistema puesto que en este caso la competición sería por el oxígeno con las bacterias oxidantes de amonio y ésta sería pues la razón por la cual éstas se ven impulsadas a crecer en suspensión.

Finalmente, el **Capítulo 8** se centra en la identificación mediante la técnica FISH de organismos de tipo filamentoso que se observaron en repetidas ocasiones durante el análisis de las poblaciones microbianas presentes en los gránulos aerobios de diferentes reactores. La caracterización de los organismos filamentosos es de gran importancia puesto que son los responsables de múltiples episodios de abultamiento y espumamiento en los sistemas de tratamiento basados en lodos activos. En el caso de los gránulos aerobios, se piensa que pueden tener una función estructural al formar parte del esqueleto del gránulo, pero aún así, su presencia puede causar problemas en la sedimentabilidad y provocar incluso la ruptura de los gránulos. En este capítulo se analizaron tres reactores granulares en los que se trataban tres tipos diferentes de agua residual: el efluente de una industria conservera (R1), el efluente de una industria de procesado de productos marinos (R2) y un medio sintético al que se le añadió coagulante y floculante (R3). El uso de la técnica FISH permitió identificar y visualizar los siguientes organismos filamentosos: *Thiothrix* y *Chloroflexi* en el reactor R1, *Meganema perideroedes* en el reactor R2 y *Chloroflexi* y *Sphaerotilus natans* en el reactor R3. Además de presentar las condiciones operacionales de cada uno de los reactores, se comentan las posibles causas y factores que pueden promover el desarrollo excesivo de estos organismos en los sistemas de tratamiento de agua residual.

Resumo

As diversas lexislacións tanto a nivel estatal como comunitario son cada vez máis esixentes en canto aos niveis de contaminantes nos efluentes industriais e urbanos. Este feito xunto coa redución das superficies dispoñibles para a súa instalación está a impulsar o desenvolvemento de novos sistemas e tecnoloxías para o tratamento das augas residuais máis eficaces e compactos nos cales se poida levar a cabo a eliminación conxunta de materia orgánica e nutrientes como o nitróxeno e o fósforo.

O emprego de reactores secuenciais (SBR: Sequencing Batch Reactors) con biomasa granular aerobia aplicados ao tratamento biolóxico de augas residuais en condicións aerobias preséntase como unha alternativa ao proceso convencional de lamas activas. Estes reactores opéranse en forma secuencial en ciclos que comprenden fases de enchido, reacción, sedimentación e baleirado. Os ciclos de operación caracterízanse pola curta duración das fases de enchido e de sedimentación co fin de desenvolver en condicións aerobias a biomasa en forma de gránulos.

Estes sistemas permiten tratar cargas máis elevadas que os sistemas de lamas activas debido á obtención de grandes concentracións de biomasa. Doutra banda, as boas características de sedimentabilidade que presentan os gránulos aerobios e a baixa produtividade de biomasa permiten prescindir dos grandes e custosos sedimentadores secundarios e, polo tanto, requírese menos espazo para a súa implantación. Outra das vantaxes destes sistemas granulares é que neles se poden levar a cabo á vez os procesos de eliminación da materia orgánica e de nitróxeno por medio dos procesos de nitrificación e desnitrificación simultánea (SND). Desta forma lógrase non só mellorar a calidade dos efluentes xerados senón tamén reducir os custos de implantación e de xestión de lodos.

Os primeiros estudos centrados na granulación de biomasa aerobia desenvolvéronse en reactores a escala laboratorio utilizando medios sintéticos para poder comprender mellor todas as variables que afectaban ao proceso de formación. Desta forma determinouse que entre os factores clave da operación destes sistemas atópanse o tempo de decantación, as forzas de estrés xeradas polas condicións hidrodinámicas ou mecánicas, o tipo de substrato, a concentración de materia orgánica (DQO) e de nitróxeno, a concentración de osíxeno no medio líquido ou a presenza de posibles sustancias inhibitorias.

O coñecemento das condicións óptimas de operación para a formación dos gránulos permitiu reducir o tempo de posta en marcha destes sistemas. A partir de aquí, as liñas de investigación neste campo diversificáronse co paso dos anos cara a temas máis específicos

que van desde o estudo do efecto da hidrodinámica nos reactores, as limitacións en canto á transferencia de masa ou o estudo das características dos gránulos tras períodos longos de almacenamento. Na maioría dos casos os traballos realizáronse empregando un medio sintético.

Co fin de avaliar a aplicabilidade desta tecnoloxía para tratar efluentes industriais realizáronse estudos en reactores a escala laboratorio e máis tarde a escala planta piloto. Esta información é imprescindible para poder realizar o escalado do sistema a nivel industrial, posto que esta é a finalidade derradeira do deseño das novas tecnoloxías.

Usando como base os coñecementos existentes (**Capítulo 1**), na presente tese estudouse a posta en marcha e operación de reactores granulares aerobios empregando dous tipos diferentes de auga residual co fin de estudar a eliminación de materia orgánica e nitróxeno xunto coas propiedades físicas dos gránulos xerados. Os efluentes estudados obtivéronse dunha industria conserveira de produtos mariños (**Capítulo 3**) e dunha granxa de produción de gando porcino (**Capítulos 5** e **6**) posto que son dous efluentes xerados por mor de actividades económicas de gran importancia en Galicia. As características comúns destas dúas correntes son o gran volume de auga residual a tratar e as elevadas concentracións de materia orgánica e nitróxeno que conteñen.

Cando se traballa con sistemas biolóxicos é moi importante considerar estes sistemas non como caixas negras senón prestar atención aos organismos implicados nos diferentes procesos. As técnicas de bioloxía molecular como a DGGE (denaturing gradient gel electrophoresis), construción de librarías de clons, FISH (fluorescent *in situ* hibrydization) permiten identificar e cuantificar aos microorganismos, así como seguir a súa dinámica poboacional ao longo da operación dos sistemas biolóxicos. Estas técnicas de bioloxía molecular empregáronse para identificar as principais poboacións microbianas que levaban a cabo os procesos biolóxicos dos reactores usados (**Capítulos 4, 7 e 8**).

Os contidos principais de cada un dos capítulos da presente tese e os obxectivos alcanzados son os seguintes:

No **Capítulo 1** preséntase unha revisión bibliográfica dos diferentes actores implicados na depuración de augas residuais. Por unha banda indícanse os motivos conducentes á necesidade do establecemento de sistemas de tratamento. A continuación faise unha breve descrición dos procesos biolóxicos de eliminación de materia orgánica e nitróxeno nas augas residuais incluíndo aspectos como a estequiometría, parámetros cinéticos ou o efecto inhibitorio de determinados compostos. Preséntase tamén información relativa aos sistemas de granulación aerobia e o seu estado da arte, incluíndo os estudos realizados ata a data tratando auga residual de orixe industrial ou urbana a escala laboratorio e os levados a cabo en instalacións a escala planta piloto. Finalmente, revísanse as principais poboacións bacterianas implicadas nos procesos biolóxicos de eliminación de nitróxeno (bacterias

oxidantes de amonio, bacterias oxidantes de nitrito, bacterias anammox e bacterias desnitrificantes) facendo unha breve descrición dos aspectos máis relevantes relacionados coa súa actividade.

No **Capítulo 2** desenvólvense os materiais e métodos utilizados ao longo dos experimentos realizados nos capítulos posteriores. Isto inclúe, tanto os parámetros convencionais de caracterización da fase líquida das augas residuais (materia orgánica, compostos nitroxenados, pH, osíxeno disolto e compostos de carbono), como a caracterización da biomasa granular desde o punto de vista das súas propiedades físicas e actividades biolóxicas, ata o das poboacións microbianas presentes na mesma. Por unha banda determináronse parámetros como a densidade dos gránulos, o índice volumétrico de lamas e empregáronse técnicas baseadas na observación con estereomicroscopio para a estimación do diámetro medio e a distribución de tamaños de partícula. Doutra banda detállanse as técnicas de bioloxía molecular empregadas para a identificación das distintas poboacións bacterianas e que se basean na amplificación de ADN, illamento e secuenciación. Preséntanse os distintos cebadores usados para a amplificación do xene 16S do ARN ribosómico e do xene amoA, que codifica a encima amonio monoosixenasa, o modo en que se aplicou a técnica DGGE e o procedemento para a construción de librarías de clons para obter a separación dos distintos fragmentos de ADN. Ademais descríbese o protocolo usado para aplicar a técnica FISH xunto cunha clasificación detallada de todas as sondas que se aplicaron ao longo dos distintos capítulos desta tese. Inclúese así mesmo no presente capítulo o procedemento para o cálculo de certos parámetros co fin de avaliar o funcionamento dos reactores operados.

No **Capítulo 3** estúdase a posta en marcha dun reactor granular aerobio para tratar auga residual procedente dunha industria conserveira de productos mariños. Na Comunidade de Galicia estas industrias, especialmente as situadas en zonas costeiras, adóitanse caracterizar pola pouca dispoñibilidade de espazo para a instalación dun sistema de tratamento convencional de augas residuais, a variabilidade na composición e a presenza no efluente producido de sustancias potencialmente inhibitorias como cloruros. Polas súas vantaxes, o uso de sistemas granulares sería unha alternativa viable para o tratamento destes efluentes. Neste capítulo empregouse un reactor SBR con biomasa granular en condicións aerobias a escala laboratorio (1,5 L) para tratar o efluente dunha planta conserveira localizada na ría de Vigo. Esta corrente presentaba un contido elevado de cloruros, procedentes do uso de auga de mar no proceso industrial, susceptibles de afectar á actividade biolóxica, polo que a posta en marcha do sistema realizouse aplicando diferentes dilucións con auga. Observouse que a pesar de que o proceso de granulación foi máis lento que nos sistemas que se alimentaban cun medio sintético, ao cabo de 75 días de operación os gránulos eran estables. Neste punto os gránulos obtidos presentaban un diámetro de 3,2 mm, un índice volumétrico de lamas de 30 mL/g SST e unha concentración de sólidos de 12 g SSV/L. O factor de dilución aplicado á alimentación foise diminuíndo progresivamente e o

sistema puido tratar de forma simultánea unha velocidade de carga orgánica de 1,46 kg DQO/(m^3 d) e unha velocidade de carga nitroxenada de 0,18 kg N/(m^3 d) con eficacias de eliminación do 95% e 40%, respectivamente. Avaliouse tamén a evolución dos perfís de concentración de materia orgánica e nitróxeno ao longo de varios ciclos de operación correspondentes ás distintas condicións operacionais. Finalmente, mediante ensaios de respirometría, comprobouse como a actividade nitrificante específica dos gránulos está íntimamente relacionada co seu diámetro. Así, a maior diámetro a actividade nitrificante específica é menor debido a maiores limitacións na transferencia de masa.

No **Capítulo 4** realízase a caracterización e o seguimento das principais poboacións bacterianas presentes na biomasa do reactor granular mediante o uso de técnicas moleculares. A técnica DGGE mostrou a evolución das poboacións microbianas desde a posta en marcha ata o final da operación do reactor avaliadas a partir da presenza ou ausencia de bandas características. A secuenciación das bandas máis representativas permitiu identificar aos xéneros *Bacteroidetes* e *Thauera* como os representativos das primeiras etapas de operación, e *Thiothrix* e *Chloroflexi* como os representativos das últimas etapas. A construción da libraría de clons permitiu identificar as principais bacterias oxidantes de amonio como pertencentes ao xénero *Nitrosomonas* e tamén outros membros da poboación heterótrofa como pertencentes á familia *Comamonadaceae* e distintos xéneros da familia *Rhodocyclaceae*. Nos gránulos aerobios analizados identificáronse dous xéneros diferentes de bacterias filamentosas, *Chloroflexi* e *Thiothrix*, que poderían actuar como un esqueleto no proceso de formación do gránulo. A aplicación da técnica FISH sobre microcortes dos gránulos permitiu visualizar a distribución espacial das bacterias oxidantes de amonio e as bacterias do xénero *Thiothrix*.

No **Capítulo 5** estúdase a posta en marcha dun reactor granular para tratar a fracción líquida do xurro xerado nunha granxa porcina situada en Santiago de Compostela. Os xurros de porco caracterízanse polo seu elevado contido en materia orgánica e nitróxeno debido ao material fecal e os ouriños dos animais. A aplicación destes efluentes sobre os terreos agrícolas como medio fertilizante é unha alternativa para a súa xestión pero non é unha solución válida cando a extensión de terreo dispoñible é limitada xa que hai que considerar cuestións como a sobrefertilización e que a lexislación vixente marca o valor máximo de aplicación en 170 kg N/Ha de terreo, o que fai necesario por tanto un tratamento, previo á vertedura, destes xurros. Na actualidade está a potenciarse a dixestión anaerobia como alternativa de valorización, xa que permite o seu aproveitamento enerxético, pero ten a desvantaxe da súa baixa eficacia en canto á eliminación de nitróxeno. O emprego de sistemas granulares aerobios permitiría a eliminación simultánea de nitróxeno e materia orgánica. Ademais o seu deseño compacto e a súa fácil operación faríano adecuado para a súa instalación nas granxas gandeiras. Para avaliar a idoneidade dos sistemas granulares usouse un reactor cun volume de 1,5 L alimentado coa fracción líquida de xurro de porco diluída con auga. Os primeiros gránulos aerobios observáronse ao cabo de 15 días de operación e

mantivéronse estables durante todo o proceso. Como exemplo no día 140 de operación a concentración de sólidos no interior do reactor era de 13 g SST/L e os gránulos aerobios tiñan un diámetro medio de 5 mm, un índice volumétrico de lamas de 32 mL/g SST e unha densidade ao redor de 55 g SSV/$L_{gránulo}$. Estudouse a estabilidade do sistema a medida que se aumentaba a velocidade de carga a tratar. Con valores de carga aplicada de 4,4 kg DQO/(m^3 d) e 0,83 kg N/(m^3 d) o sistema eliminaba simultaneamente o 87% da materia orgánica e o 70% de nitróxeno. Para poder tratar o xurro sen necesidade de dilución previa efectuouse unha redución do volume de intercambio o que produciu o crecemento excesivo de biomasa en suspensión con propiedades de sedimentabilidade peores que a biomasa granular. Con todo comprobouse que o sistema seguía eliminando con eficacia similar a materia orgánica con cargas de ata 7,0 kg DQO/(m^3 d) mentres que a eliminación de nitróxeno viuse reducida polo que se fixo necesaria a aplicación dun post-tratamento para a eliminación de nitróxeno.

No **Capítulo 6** estúdase a viabilidade da aplicación do proceso CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) para tratar o efluente do reactor granular alimentado con xurro sen diluír, posto que esa corrente de saída, e de forma análoga a dos sistemas de dixestión anaerobia, caracterizábase por unha baixa concentración de materia orgánica e un elevado contido de nitróxeno (baixa relación DQO/N). Nestes sistemas CANON coexisten dous grupos de bacterias autótrofas, as bacterias oxidantes de amonio e as bacterias anammox, de forma que as primeiras oxidan parte do amonio a nitrito e as últimas combinan ambos os para producir nitróxeno gas. Aínda que o proceso anammox xa foi empregado anteriormente con efluentes da dixestión anaerobia de xurros este traballo é un dos primeiros nos que se usa o proceso CANON a baixa temperatura para o seu post-tratamento. Para este estudo empregouse reactor cun volume de 1,5 L ao que se proporciona o osíxeno mediante pulsos de aire co fin de manter a concentración de osíxeno no medio líquido nos valores axeitados para as bacterias oxidantes de amonio, non afectar ás bacterias anammox e favorecer a mestura e a transferencia de materia no líquido. Neste caso avaliouse a capacidade do sistema para tratar o efluente producido no reactor granular aerobio, chegando a unha velocidade de carga aplicada de 1,25 kg N/(m^3 d) e a unha eficacia de eliminación do 75%. Neste capítulo realízase así mesmo un estudo comparativo da aplicación do sistema CANON con outros procesos que se propuxeron nos últimos anos para a eliminación de nitróxeno dos efluentes gandeiros.

No **Capítulo 7** profúndase nas características físicas e na caracterización das poboacións microbianas da biomasa presente no reactor CANON. Preténdese avaliar a coexistencia entre as bacterias heterótrofas, que se poderían desenvolver debido á presenza de materia orgánica e as condicións microaerobias utilizadas, e as bacterias autótrofas (anammox e oxidantes de amonio). A biomasa deste reactor desenvolveuse distribuída en dúas fraccións: unha en forma de gránulos cun diámetro medio de 2,6 mm e cunha cor avermellada intensa, debida á presenza de bacterias anammox, e outra en suspensión, composta principalmente por bacterias oxidantes de amonio e heterótrofas. Mediante a realización dunha libraría de

clons e a aplicación da técnica de DGGE identificáronse membros do filum *Bacteroidetes* e da familia *Comamonadaceae* e do xénero *Zoogloea* pertencentes á poboación de bacterias heterótrofas. O uso de cebadores específicos para a determinación da poboación anammox revelou a presenza de bacterias pertencentes ao xénero *Candidatus* "Brocadia anammoximans" e *Candidatus* "Brocadia fulgida". Finalmente, identificáronse as bacterias oxidantes de amonio, que pertencían ao xénero *Nitrosomonas*. O uso da técnica FISH permitiu realizar unha estimación da abundancia dos principais organismos implicados no proceso CANON, cunha abundancia relativa do 35% de bacterias oxidantes de amonio e 30% de anammox. A presenza de bacterias heterótrofas en sistemas anammox podería ser a responsable de problemas de competición por substratos comúns como o nitrito para desnitrificar. Con todo, e debido ás condicións de operación do reactor, non se observou a desestabilización do sistema posto que neste caso a competición sería polo osíxeno coas bacterias oxidantes de amonio e esta sería a posible razón pola cal estas bacterias se ven impulsadas a crecer en suspensión.

Finalmente, o **Capítulo 8** céntrase na identificación mediante a técnica FISH de organismos de tipo filamentoso que se observaron en repetidas ocasións durante a análise das poboacións microbianas presentes nos gránulos aerobios de diferentes reactores. A caracterización dos organismos filamentosos é de gran importancia posto que son os responsables de múltiples episodios de avultamento e escumamento nos sistemas de tratamento baseados en lamas activas. No caso dos gránulos aerobios, pénsase que poden ter unha función estrutural ao forman parte do esqueleto do gránulo, pero aínda así, a súa presenza pode causar problemas na sedimentabilidade e provocar incluso a rotura dos gránulos. Neste capítulo analizáronse tres reactores granulares nos que se trataban tres tipos diferentes de auga residual: o efluente dunha industria conserveira (R1), o efluente dunha industria de procesado de produtos mariños (R2) e un medio sintético ao que se lle engadiu coagulante e floculante (R3). O uso da técnica FISH permitiu identificar e visualizar os seguintes organismos filamentosos: *Thiothrix* e *Chloroflexi* no reactor R1, *Meganema perideroedes* no reactor R2 e *Chloroflexi* e *Sphaerotilus natans* no reactor R3. Ademais de presentar as condicións operacionais de cada un dos reactores, coméntanse as posibles causas e factores que poden promover o desenvolvemento excesivo destes organismos nos sistemas de tratamento de auga residual.

Summary

The new regulations and policies, both at a State and European Community level, are the more and more exigent regarding the concentrations of pollutants in the generated effluents at industrial and urban level. This fact, together with the reduction of the available surfaces for their management, is promoting the development of new more effective and compact systems and technologies for wastewater treatment where the simultaneous organic matter and nutrients removal, like nitrogen and phosphorus, can be performed.

The use of sequencing batch reactors (SBR) with aerobic granular biomass applied for the biological wastewater treatment in aerobic conditions is presented as an alternative to the conventional activated sludge processes. These reactors are operated in sequential cycles that comprise filling, reaction, settling and withdrawal phases that are characterised by the short length of the filling and settling ones aiming to the development of biomass in the shape of granules in aerobic conditions.

These systems allow the treatment of higher loading rates than the activated sludge systems because higher biomass concentrations can be achieved. On the other hand, the excellent settling properties of aerobic granules and their low biomass productivity allow doing without the big and expensive secondary settlers and moreover smaller footprint is required for its implantation. Another advantage of these granular systems is that organic matter and nitrogen removal can be simultaneously carried out in the same unit by means of simultaneous nitrification and denitrification processes (SND). In this way not only the improvement of the quality of the generated effluents but also a reduction of the implementation and sludge management costs are attained.

First studies focused on aerobic granulation were developed at laboratory scale reactors using synthetic media in order to better understand all the variables that affected the granulation process. In this way the settling time, the hydrodynamic shear and stress forces, the type of substrate, the organic matter (COD) and nitrogen concentrations, the dissolved oxygen concentration in the bulk liquid or the presence of possible inhibitory substances were identified to be among the key factors affecting the operation of these systems.

The knowledge of the optimal operational conditions for the formation of granules allowed the reduction of the start-up time of these systems. From here, the lines of research in this field have been diversified during time into more specific subjects that go from the study of the effect of the stress conditions, the mass transfer limitations or the study of the

characteristics of the granules after long periods of storage. In most of the cases the works have been carried out using a synthetic medium.

With the aim of evaluating the applicability of this technology to treat effluents from different industrial sectors studies treating wastewater in reactors, at laboratory scale and later at pilot plant scale reactors, have been performed. This information is indispensable in order to carry out the scale up of these systems to industrial level, since this is the last purpose of the design of new technologies.

Taking the existent knowledge as starting point (**Chapter 1**), in the present thesis the start up and the performance of aerobic granular reactors employing two different types of wastewater were studied. Special attention was paid to organic matter and nitrogen removal together with the physical properties of the generated granules. The studied effluents were obtained from a fish canning industry (**Chapter 3**) and from a pig farm (**Chapters 5** and **6**) since these are two types of effluents generated as a result of economic activities of great importance in Galicia. The common characteristics of these two effluents are the large volume of wastewater to be treated and the high contained organic matter and nitrogen concentrations.

When working with biological systems it is very important not only to consider them as black boxes but also to pay attention to the organisms involved in the different processes. Molecular biology techniques like the DGGE (denaturing gradient gel electrophoresis), clone libraries and FISH (fluorescent *in situ* hibrydization) allow identifying and quantifying the microorganisms, as well as to follow their populations dynamics during the operation of the biological systems. These techniques of molecular biology were used to identify the main microbial populations that carried out the biological processes in the used reactors (**Chapters 4, 7** and **8**).

The main contents of each one of the chapters of the present thesis and the achieved objectives are detailed in the following sections:

In **Chapter 1**, a bibliographic review of the different actors involved in wastewater treatment processes is presented. First, the reasons that drive to the need of the establishment of wastewater treatment systems are also indicated. Later a brief description is provided of the biological processes for organic matter and nitrogen removal from wastewater, including aspects as stoichiometry, kinetic parameters or the inhibitory effect of certain compounds. Information related to aerobic granular systems and its state of the art, including studies performed up to date treating industrial or urban wastewater at laboratory and pilot plant scale, is presented. Finally, the main bacterial populations involved in the nitrogen removal biological processes (ammonia oxidizing, nitrite oxidizing, anammox and denitrifying bacteria) are indicated, providing with a brief description of the most relevant aspects related to their activity.

In **Chapter 2,** the material and methods, used in the different experiments performed along the following chapters, are described. Either conventional parameters used for the characterisation of the wastewater liquid fraction (organic matter, nitrogenous compounds, pH, dissolved oxygen and carbon compounds), or those used for the characterisation of the granular biomass from its physical properties and biological activities to the present microbial populations. On one hand parameters like granules density and sludge volume index were determined and techniques based on the observation under a stereomicroscope for the estimation of particles average diameter and size distribution were used. On the other hand, molecular biology techniques used for the identification of the different bacterial populations based on the DNA amplification, isolation and sequencing are described. The primers used for the amplification of the 16S ribosomal RNA and the amoA gene, the way of use of the DGGE technique and the procedure for the cloning libraries construction, to obtain the separation of the different fragments of DNA, are specified. Furthermore the used protocol for the application of the FISH technique together with a detailed classification of all the probes that have been applied during the distinct chapters of this thesis are described. In this chapter the procedure for the calculation of some parameters with the aim of evaluating the operation of the utilized reactors is also included.

In **Chapter 3**, the start up and performance of a granular aerobic SBR reactor treating the wastewater from a fish canning industry was studied. In the Community of Galicia these industries, especially those situated in coastal zones, are characterised by the low space availability for the installation of a conventional system for wastewater treatment, the variability in the composition and the presence of potentially inhibitory substances like chlorides in the produced effluent. Due to their advantages, the use of granular systems would be a viable alternative for the treatment of these effluents. In this chapter a SBR reactor with aerobic granular biomass was operated at scale laboratory (1.5 L) to treat the effluent from a fish canning industry located in the Ria of Vigo. This effluent presented a high concentration of chlorides due to the use of sea water in the industrial process which could affect the biological activity of the treatment system. Therefore, the start up of the process was performed by applying different dilutions with tap water. Although the time needed to achieve complete granulation was longer than that previously reported in systems using a synthetic medium, after 75 days of operation, the granules were stable. At this moment the obtained granules had a diameter of 3.2 mm, a sludge volumetric index of 30 mL/g TSS and a solids concentration of 12 g VSS/L. The applied dilution factor to the feeding was progressively diminished and the system could treat simultaneously an organic loading rate of 1.46 kg COD/(m^3 d) and a nitrogen load rate of 0.18 kg N/(m^3 d) with removal efficiencies of 95% and 40%, respectively. The evolution of the concentration profiles of organic matter and nitrogen during the operational cycles from distinct operational stages was evaluated. Finally, by means of respirometric batch assays, a close relation between the nitrifying activity of the granules and their diameters was proved. Therefore, the larger the diameter of the granules the lower the nitrifying specific activity of the biomass, due to mass transfer limitations.

In **Chapter 4**, the characterisation and monitoring of the main bacterial populations present in the biomass of the granular reactor was carried out by means of the use of molecular techniques. The DGGE technique showed the evolution of the microbial populations from the start up to the end of the operation of the reactor. The evaluation was performed according to the presence or absence of characteristic bands. The sequencing of the most representative bands allowed identifying the genus *Bacteroidetes* and *Thauera* as the representatives of the first stages of operation, and *Thiothrix* and *Chloroflexi* as the representatives of the last stages. The construction of the clone library allowed the identification of the genus *Nitrosomonas* as the main ammonia oxidizing bacteria, and also other members of the heterotrophic population, that belonged to the family *Comamonadaceae* and to different genera of the *Rhodocyclaceae* family. Two different genera of filamentous bacteria, *Chloroflexi* and *Thiothrix*, were also identified in the aerobic granules. They could act like a skeleton in the granule formation process. The application of the FISH technique to cryosectioned granules allowed to visualise the spatial distribution of the ammonia oxidizing and *Thiothrix* genera bacteria.

In **Chapter 5**, the start up and performance of a granular reactor to treat the liquid fraction of the slurry generated in a swine farm located in Santiago de Compostela was studied. Swine slurry is characterised by its high organic matter and nitrogen concentration because of the faecal material and the urine of the animals. The application of these effluents on the crop lands as a fertilizer is an alternative for its management but is not a valid solution when the extension of available land to spread them is limited. It is necessary to consider questions like the over fertilization and that the current legislation marks the maximum value of application in 170 kg N/ha/year, and this makes a treatment of slurries prior to be spread necessary. Nowadays, the anaerobic digestion is being boosted as an alternative for its valorisation because of the energetic use of the produced biogas, but it has the disadvantage of its low efficiency regarding the nitrogen removal. The use of aerobic granular systems would allow the simultaneous nitrogen and organic matter removal. Furthermore their compact design and easy operation would make them appropriate for its installation in the farms. A SBR reactor with a volume of 1.5 L, fed with the liquid fraction of swine slurry diluted with tap water, was used in order to evaluate the suitability of the granular systems. Aerobic granules were observed after 15 days of operation and were stable during the whole reactor operation. As an example, on the day 140 of operation, the solids concentration inside the reactor was of 13 g TSS/L and the aerobic granules had an average diameter of 5 mm, a sludge volumetric index of 32 mL/g TSS and a density around 55 g VSS/$L_{granule}$. The stability of the system while the applied loading rate increased was studied. When 4.4 kg COD/(m^3 d) and 0.83 kg N/(m^3 d) were applied, the system removed 87% of the organic matter and 70% of nitrogen simultaneously. In order to treat the swine slurry without previous dilution the volumetric exchange ratio was reduced, but this promoted the excessive growth of dispersed biomass with worse settling properties than the granular biomass. However it was checked that the system continued with a similar efficiency in terms of organic matter removal when

loads up to 7.0 kg COD/(m^3 d) were applied whereas the elimination of nitrogen was reduced, making necessary the application of a post-treatment for nitrogen removal.

In **Chapter 6**, the feasibility of the application of the CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) process to treat the effluent generated in the aerobic granular reactor fed with swine slurry was studied. This wastewater, with a similar composition to those generated in anaerobic digesters, was characterised by a low organic matter concentration and high nitrogen content (low COD/N ratio). Two groups of autotrophic bacteria, ammonia oxidizing and anammox bacteria, coexist in the CANON systems, so the former oxidise part of the ammonium to nitrite and the later combine both to produce nitrogen gas. Even though the anammox process has been previously used with effluents from anaerobic digesters treating swine slurry, the present work is one of the first in which the CANON process has been used as a post-treatment of swine slurry operated at low temperature. A SBR reactor with a volume of 1.5 L was used. It was characterized by the use of pulsed air to maintain the oxygen concentration in the bulk liquid at the values accurate for the ammonia oxidizing and anammox bacteria and also favour the mixing and the mass transfer in the bulk liquid. In this case, the capacity of the system to treat the effluent produced in the aerobic granular reactor was evaluated, with an applied nitrogen loading rate of 1.25 kg N/(m^3 d), with a removal efficiency of 75%. In this chapter a comparative study of the applicability of the CANON system with other processes that have been posed in the last years for nitrogen removal from livestock effluents is performed.

In **Chapter 7**, an insight of the physical properties and microbial populations characterization of the biomass from the CANON reactor is carried out. The coexistence between the heterotrophic bacteria, which could develop because of the presence of organic matter and the microaerobic conditions, and the autotrophic bacteria (anammox and ammonia oxidizing bacteria) is intended to be evaluated. The biomass from this reactor was distributed in two fractions: one fraction in the form of granules with an average feret diameter of 2.6 mm and with an intense reddish colour, because of the presence of anammox bacteria, and another one in the form of dispersed biomass, mainly composed by ammonia oxidizing and heterotrophic bacteria. By means of the realisation of a clone library and the application of the DGGE technique, members of the phylum *Bacteroidetes*, the family *Comamonadaceae* and the genus *Zoogloea* were identified as members of the heterotrophic bacteria population. The use of specific primers for the identification of the anammox population revealed the presence of bacteria that belonged to the genus *Candidatus* “Brocadia anammoximans” and *Candidatus* “Brocadia fulgida”. Finally, ammonia oxidizing bacteria were identified to belong to the genus *Nitrosomonas*. The use of the FISH technique allowed an estimation of the abundance of the main organisms involved in the CANON process, with a relative abundance of 35% of ammonia oxidizing bacteria and 30% of anammox. The presence of heterotrophic bacteria in anammox systems could be responsible for the events of competition for the common substrates like the nitrite for denitrification.

However, thanks to the operational conditions of this reactor the destabilization of the system was not observed since the competition would be established for the oxygen with the ammonia oxidizing bacteria. This would be the reason why these organisms are forced to grow in suspension.

Finally, **Chapter 8** was focused on the identification, by means of the FISH technique, of filamentous organisms that were observed in repeated occasions when the analysis of the microbial populations present in the aerobic granules of different reactors was performed. The characterisation of the filamentous organisms is of great interest since they are responsible of multiple episodes of bulking and foaming in wastewater treatment systems based on activated sludge. In the case of aerobic granules, it is thought that they can have a structural function and form part of the skeleton of the granule, but, its presence can cause settleability problems and even cause the breakage of the granules. In this chapter three granular reactors treating three different types of wastewater were analysed: the effluent from a fish canning industry (R1), the effluent from a plant processing marine products (R2) and a synthetic medium supplemented with coagulant and floculant (R3). The use of the FISH technique allowed identifying and visualising the following filamentous organisms: *Thiothrix* and *Chloroflexi* in the reactor R1, *Meganema perideroedes* in the reactor R2 and *Chloroflexi* and *Sphaerotilus natans* in the reactor R3. The operational conditions of each one of the reactors and also the possible causes and factors that can promote the excessive development of these organisms in wastewater treatment systems were described.

Chapter 1:

Introduction

Summary

The scope of this chapter is to present answers to some basic questions related to the field of the biological wastewater treatment. Organic matter, nutrients such as nitrogen and phosphorus cause eutrophication in natural media, leading to a significant negative impact to aqueous environment, but also other contaminant compounds such as metals, inorganic compounds etc. can negatively affect natural environments. These are the reasons "why" pollutants removal from municipal and industrial wastewater is strongly required.

Biological processes are cost-effective and widely adopted in the world for nutrients removal from wastewater. A brief description of "how" the biological process of organic matter and nitrogen removal work is also provided. Special attention is given to new processes such as partial nitritation and anammox and also to a new technology based in the use of aerobic granules in sequencing batch reactors "where" the previously described processes can be carried out.

The state of the art of aerobic granulation and also recent studies carried out with industrial and urban wastewater and also the last finding in removal of toxic compounds are presented to identify "what for" are the biological processes used.

The final part of this section is centred in the main players "which" perform the most important part of all the biological processes in wastewater treatment: the microorganisms. Therefore, heterotrophic and autotrophic bacteria related to nitrogen removal processes are presented.

1.1. WHY?: WATER POLLUTION REMOVAL

Humans have settled near water bodies for millennia, and human alteration of coastlines, rivers, lakes and wetlands has gone hand-in-hand with social and economic development. While this situation has increased the demand of the amount of food production, urban growth and industrial development have also pushed the cities to look further for the increasing amount of the water they need and to take care of the wastewater they produce. Wastewater is generated either in households or from the industrial activity. In one way or another it can get into waters of closed reservoirs, rivers, seas and oceans where a whole variety of harmful substances are being accumulated.

But the concern about wastewater is not new: historical records show that sewage collection systems have existed for thousands of years, with Babylonians and Assyrians, and later the Romans. For example, the Romans developed an organized and centralized system of aqueducts, siphons and collection systems of used water. One example is the "Cloaca Maxima", a sewage system constructed in the ancient Rome around 600 BC in order to drain local marshes, remove the waste and carry the effluents to the River Tiber which flow next to the city.

Unfortunately, medieval communities were not so aware about the problematic of the wastewater and intensive construction of drainage systems in Europe did not begin until the 19th century. Until then, the sole existing options were the sewers consisting in open ditches that conducted the wastewaters from the households towards the rivers and eventually out to the sea. As an example, during the early 19th century the River Thames was an open sewer, with disastrous consequences for public health in London, including numerous cholera and typhoid epidemics. In 1856 several proposals to modernize the sewerage system were studied but refused due to the lack of funds for implementation. However, after The Great Stink (or the Big Stink) in the summer of 1858, during which the smell of untreated human waste was very strong in central London, the Parliament realized about the urgency of solving the problem and resolved to create a modern sewerage system. At this time the London Metropolitan Board of Works, was given the responsibility for the work. An extensive underground sewerage system that diverted waste to the Thames Estuary was designed and six main intercepting sewers were constructed. When the project was completed London had 2100 km of sewers.

Few years later a law on sewage purification from faecal and decomposing substances previous to their discharge into rivers was adopted in England. In 1898 the Royal Commission on Sewage Disposal was created, with the purpose of coordinating the evaluation of new treatment process. The earliest sewage detoxication methods were based on the use of irrigated or filtration fields. Therefore, sewage utilization in agriculture and its purification were coupled in one process in the irrigated farm fields. The land application of these wastes in the right quantities to allow sufficient aeration and drainage offered a method for the rapid

stabilization of the organic material. Due to the large surface area requirements of this treatment its application to the sewage produced in large modern cities was impracticable.

A range of simple small scale biological treatment systems including cesspits, septic tanks, land effluent percolation and trickling filters, all operating on a small scale had been introduced by that time. Although these systems were slow and inefficient to treat the huge volumes of generated wastewater, they settled the basis of the wastewater treatment systems that are in use to this day (i.e. the activated sludge process that was developed in 1914). But this treatment systems were not sufficient and the population growth and the rapid industrialization which took place during the twentieth century led to the generation of vast amounts of wastewater that was introduced into the environment. This has affected the ecosystems and also has caused health problems for the inhabitants residing near the factories. As people became more aware of the effects of the wastewater on nature, due to the indiscriminate discharge of pollutants, and as new laws imposed more stringent discharge norms, efforts have been made to treat them so as to make them innocuous before discharge.

The composition of the sewage in 19th century differs from the effluents generated nowadays mainly in the presence of chemicals. Water pollution is considered as any chemical, physical or biological change in the quality of the water that has a harmful effect on any living being that drinks or uses or lives in it. Several classes of water pollutants are usually considered, e.g.:

- Suspended solids: Suspended solids can lead to the development of sludge deposits and anaerobic conditions when untreated wastewater is discharged in the aquatic environment.
- Biodegradable organics: Composed principally of proteins, carbohydrates and fats. Biodegradable organics are measured mostly in terms of BOD (biochemical oxygen demand) and COD (chemical oxygen demand). If these are discharged untreated to the environment, their subsequent natural biological stabilization can lead to the depletion of natural oxygen resources and to the development of septic conditions.
- Pathogens: Communicable diseases can be transmitted by the pathogenic organisms that may be present in wastewater. These are bacteria, viruses, protozoa and parasitic worms.
- Nutrients: Both nitrogen and phosphorus, along with organic carbon, are essential nutrients for growth. When discharged to the aquatic environment, these nutrients can lead to the outgrowth of undesirable aquatic life. When discharged on land in excessive amounts, they can also lead to the groundwater pollution and the excessive growth of algae and other water plants, which deplete the dissolved oxygen supply in water causing the eutrophication.

- Priority pollutants: Organic and inorganic compounds selected on the basis of their known or suspected carcinogenicity, mutagenicity, teratogenicity or high acute toxicity. Many of these compounds are found in wastewater.
- Emerging contaminants: includes a diverse collection of chemical substances, therapeutic and veterinary drugs, fragrances, lotions, and cosmetics.
- Refractory organics: These organic compounds tend to resist conventional methods of wastewater treatment. Typical examples include surfactants, phenols, and agricultural pesticides.
- Heavy metals: Heavy metals are usually added to the wastewater from commercial and industrial activities and may have to be removed if the wastewater is to be reused.
- Dissolved inorganics: Inorganic constituents such as calcium, sodium and sulphate are added to the original domestic water supply as a result of water use and may have to be removed if the wastewater is to be reused.

A great number of different techniques have been worked out in field of the wastewater treatment based on physicochemical or biochemical processes to remove harmful components.

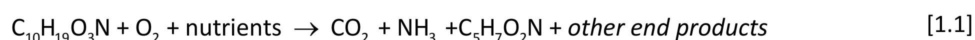
1.2. HOW?: BIOLOGICAL PROCESSES

The treatment of contaminated wastewater by means of biological and chemical processes has been widely implemented from classical urban to industrial wastewater. From economical and operational points of view, biological treatments have proved to be a robust and more energy efficient way of treating biodegradable pollutants if good process control can be ensured (Grady *et al.*, 1999). A wide variety of compounds, such as organic matter and nutrients (mainly nitrogen and phosphorus), can be biologically removed to reduce the discharge levels in water bodies (rivers, lakes and seas).

1.2.1. Organic matter oxidation

The aerobic removal of organic matter can be accomplished either in suspended or attached growth. Both require sufficient contact time between the wastewater and the heterotrophic microorganisms involved in the process, and the presence of sufficient oxygen and nutrients content. During the initial biological uptake of the organic material, more than half is oxidized and the remaining half is assimilated as new biomass, which may be further oxidized by endogenous respiration.

During the aerobic oxidation, the conversion of organic matter is carried out by mixed bacterial cultures in general accordance with the stoichiometry shown in equation 1.1:



$$C_{10}H_{19}O_3N + O_2 + \text{nutrients} \rightarrow CO_2 + NH_3 + C_5H_7O_2N + \textit{other end products} \quad [1.1]$$

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

$$C_5H_7NO_2 + 5\,O_2 \rightarrow 5\,CO_2 + 2\,H_2O + NH_3 + \textit{energy} \qquad [1.2]$$

For organic matter removal, pH in the range of 6.0 to 9.0 is tolerable, while optimal performance occurs near a neutral value. A Dissolved Oxygen (DO) concentration of 2 mg O_2/L is commonly used in activated sludge systems. Depending on the treated wastewater, care must be taken to assure that sufficient nutrients (N and P) are available for the amount of organic matter to be treated (Tchobanoglous *et al.*, 2003).

1.2.2. Nitrification

The nitrification process is carried out in two sequential stages: the ammonium oxidation to nitrite (nitritation process) and the subsequent oxidation of nitrite to nitrate (nitratation process). Nitrification is the initial step of the biological nitrogen removal processes and is carried out by two phylogenetically independent groups of autotrophic aerobic bacteria, namely, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Recently, it was discovered that ammonium oxidation can also be performed by archaea (Konneke *et al.*, 2005) that were found also to occur in wastewater treatment plants (WWTPs) operated at low dissolved oxygen levels and long solid retention times (Park *et al.*, 2006).

Hydroxylamine is produced as an intermediate in the oxidation step of ammonium to nitrite by AOB. The enzymes involved in the nitritation process are ammonia monooxygenase (amoA) that catalyzes the oxidation of ammonia to hydroxylamine (Eq. 1.3) and hydroxylamine oxidoreductase (HAO) that catalyses the oxidation of hydroxylamine to nitrite (Eq. 1.4). The complete process is described in equation 1.5.

$$NH_4^+ + O_2 + H^+ + 2\,e^- \rightarrow NH_2OH + H_2O \qquad [1.3]$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 4\,e^- + 5\,H^+ \qquad [1.4]$$

$$NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2\,H^+ \qquad [1.5]$$

In the second stage of the nitrification process, nitrite is oxidized to nitrate by NOB by means of the nitrite oxidoreductase enzyme (NOR) (Eq. 1.6).

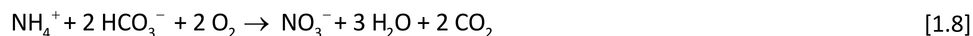
$$NO_2^- + 0.5\,O_2 \rightarrow NO_3^- \qquad [1.6]$$

The total oxidation reaction of the nitrification process is presented as shown in equation 1.7:

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O \qquad [1.7]$$

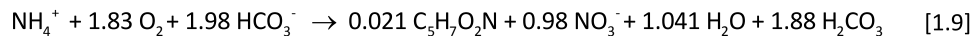
Based on the above mentioned total oxidation reaction (Eq. 1.7), the oxygen amount required for the complete oxidation of ammonia is 4.57 g O_2/g NH_4^+-N oxidized with 3.43 g O_2/g NH_4^+-N used for nitrite production and 1.14 g O_2/g NO_2^--N oxidized to nitrate.

The production of protons during the nitrification process is neutralized by the buffer capacity of the carbonate system through CO_2 stripping (Eq. 1.8).



$$NH_4^+ + 2\ HCO_3^- + 2\ O_2 \rightarrow NO_3^- + 3\ H_2O + 2\ CO_2 \qquad [1.8]$$

The whole metabolism of the bacteria including their growth (combination of anabolism and catabolism) is described by means of the following stoichiometric equation (Eq. 1.9) where the fixation of inorganic carbon and its equilibrium are presented.



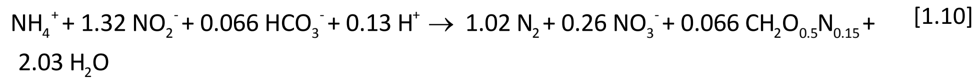
$$NH_4^+ + 1.83\ O_2 + 1.98\ HCO_3^- \rightarrow 0.021\ C_5H_7O_2N + 0.98\ NO_3^- + 1.041\ H_2O + 1.88\ H_2CO_3 \qquad [1.9]$$

From the stoichiometry of Eq. 1.9 an amount of 7.07 g alkalinity as $CaCO_3$ is required per 1 g of ammonia oxidized (NH_4^+-N) due to the release of protons during the ammonium oxidation. In those cases when this amount of buffer is not available in the water, the pH of the medium drops and the ammonium oxidation rate sharply decreases. When the synthesis is considered (Eq. 1.9), the amount of oxygen required is of 3.16 g O_2/g NH_4^+-N and 1.11 g O_2/g NO_2-N.

The nitrification process is affected by a number of environmental factors including: metals, organic and inorganic compounds, pH, un-ionized ammonia or free ammonia (NH_3, FA) and free nitrous acid (N-HNO_2, FNA). In the case of free ammonia and free nitrous acid, the inhibitory effects are dependent on the total nitrogen species concentration, the temperature and the pH value as reported by Anthonisen *et al.*, (1976).

1.2.3. Anaerobic ammonia oxidation: Anammox

For a long time it was thought that the ammonium oxidation could only take place aerobically. Broda, (1977) predicted, using thermodynamic calculations, the existence of chemolitoautotrophic bacteria capable to oxidize ammonium using nitrite as electron acceptor. That prediction would be experimentally confirmed two decades later by Mulder *et al.*, (1995) in a denitrifying pilot plant, treating wastewaters from a yeast factory, where the disappearance of nitrogen unjustified by the balance was observed. This process was called anaerobic ammonium oxidation (anammox). Anammox bacteria convert ammonium together with nitrite (electron acceptor) directly to dinitrogen gas following the reaction described in Eq. 1.10 (Strous *et al.*, 1998). In this process a small amount of nitrate is produced in the anabolism of anammox bacteria and neither oxygen nor organic matter is needed in the process.



$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad [1.10]$$

Anammox bacteria have been detected in several WWTPs all around the world. Their optimal operational temperature and pH are of 35 °C and 8, respectively. These bacteria are characterised by a low biomass productivity of 0.038 g VSS/g N and a slow growth rate with doubling times as long as 11 d (Strous *et al.*, 2002). The advantage of this low biomass productivity is the reduction of operational costs related to the sludge handling in the WWTP. On the other hand, the slow growth rate of the anammox bacteria makes the start up of the anammox process long and difficult. It is therefore mandatory to start-up the process in reactors with good biomass retention (Fernandez *et al.*, 2008).

Another important characteristic of anammox bacteria is the fact that they are inhibited by both O_2 and NO_2^-. Anammox bacteria are inhibited by an oxygen partial pressure of only 0.5% of air saturation, however, this inhibition is reversible once anaerobic conditions are re-established (Strous *et al.*, 1997). Related to NO_2^- no uniformity is found in the literature about the threshold values for inhibitory effects. For instance, Strous *et al.*, 1999b reported a value of 100 mg N/L as completely inhibitory whereas Dapena-Mora *et al.*, (2007) reported that concentrations of nitrite of 350 mg N/L corresponded only to 50% inhibition of anammox bacteria activity. Further short and long-term studies were performed to evaluate the inhibitory effects of this compound (Fernández *et al.*, ; Bettazzi *et al.*, 2010; Kimura *et al.*, 2010), but the obtained result could not clarify this point because the inhibitory levels were established in values as diverse as 30 to 150 mg NO_2^--N/L. What is known up to date is that, it is mandatory to maintain nitrite concentrations to low values in order to avoid the breakdown of the process and that the limit concentration value for normal operation depends on the used system.

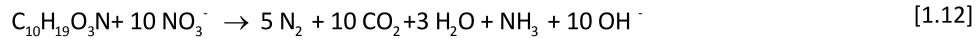
1.2.4. Denitrification

In the denitrification process, the nitrate and/or nitrite present in the wastewaters is reduced to molecular nitrogen gas in anoxic conditions (in the absence or under limited DO concentrations) by the action of heterotrophic bacteria. The process requires the presence of an organic carbon source as electron donor, e.g. acetic acid or methanol, and a nitrogen oxide (nitrate or nitrite) which acts as the last electron acceptor in the respiratory chain substituting the O_2 molecule. The reduction process is carried out by subsequent steps through nitrogen compounds in different oxidation states (Eq. 1.11).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad [1.11]$$

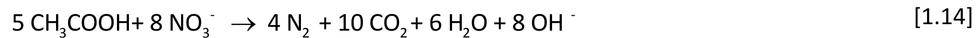
In biological nitrogen removal processes, the electron donor can be the soluble organic matter present in the treated wastewater (Eq. 1.12), the organic matter produced during

endogenous decay or an exogenous added source such as methanol or acetate (Eqs. 1.13 and 1.14).



$$C_{10}H_{19}O_3N + 10\ NO_3^- \rightarrow 5\ N_2 + 10\ CO_2 + 3\ H_2O + NH_3 + 10\ OH^- \quad [1.12]$$

$$5\ CH_3OH + 6\ NO_3^- \rightarrow 3\ N_2 + 5\ CO_2 + 7\ H_2O + 6\ OH^- \quad [1.13]$$



$$5\ CH_3COOH + 8\ NO_3^- \rightarrow 4\ N_2 + 10\ CO_2 + 6\ H_2O + 8\ OH^- \quad [1.14]$$

From the stoichiometry it can be inferred that the denitrification process originates an increase of the alkalinity of the medium of 3.57 g of alkalinity (as $CaCO_3$) per g of NO_3-N reduced. The oxygen equivalent of using nitrate as electron acceptor is of 2.86 g O_2/g NO_3-N.

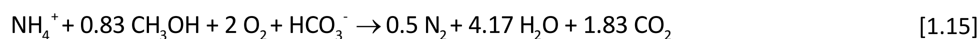
1.2.5. Coupled processes

In order to perform the nitrogen removal from wastewater, the processes previously described can be combined according to two main different configurations: nitrification-denitrification or partial nitrification-anammox.

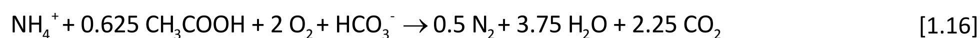
Nitrification-denitrification

Due to the different operational conditions of these processes, two different tanks are needed: one stirred but not aerated where denitrification is carried out, and another aerated, where ammonium and organic matter are simultaneously oxidized. Pre-denitrification or post-denitrification options are both possible layouts. Combined nitrification-denitrification processes are effective in maintaining neutral pH level in the reactor, without the addition of external acid/base source. Alkalinity is consumed during nitrification, but produced during denitrification.

Combining the equations obtained for the nitrification with those corresponding to the denitrification and taking into account the carbonate system equilibrium, the stoichiometry of the complete nitrogen removal by nitrification-denitrification with two different substrates is as follows (Eq. 1.15 - 1.16).



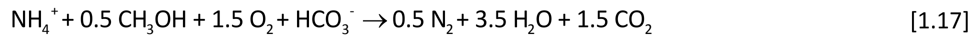
$$NH_4^+ + 0.83\ CH_3OH + 2\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 4.17\ H_2O + 1.83\ CO_2 \quad [1.15]$$



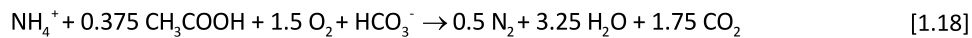
$$NH_4^+ + 0.625\ CH_3COOH + 2\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.75\ H_2O + 2.25\ CO_2 \quad [1.16]$$

In the last years, new strategies arose focused on the development of a shortcut in the nitrogen cycle named the “nitrite route”, which avoids the reaction of nitrite oxidation to nitrate (Eq. 1.6). In this case, the denitrification to nitrogen gas is performed only by using the nitrite produced by AOB. To restrict the process to the nitritation stage, the oxidation of nitrite to nitrate carried out by NOB has to be avoided making use of the different growth rates of both populations, oxygen affinity constants and/or inhibitory characteristics between AOB and NOB (Anthonisen *et al.*, 1976; Wiesmann, 1994; Garrido *et al.*, 1997; Hellinga *et al.*,

1998; Carrera *et al.*, 2004; Vázquez-Padín *et al.*, 2010b). Using the same substrates (Eq. 1.15-1.16), the stoichiometry of the nitritation-denitrification process is as follows (Eq. 1.17- 1.18):



$$NH_4^+ + 0.5\ CH_3OH + 1.5\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.5\ H_2O + 1.5\ CO_2 \quad [1.17]$$



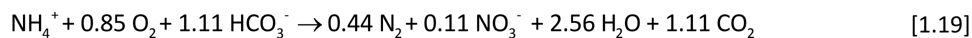
$$NH_4^+ + 0.375\ CH_3COOH + 1.5\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.25\ H_2O + 1.75\ CO_2 \quad [1.18]$$

Therefore, denitrification of nitrite to nitrogen gas results in 40% costs savings for external carbon source addition, the 25% less oxygen requirements and also less CO_2 emission than in the case of denitrification of nitrate. As in the case of denitrification with nitrate, half of the protons produced during nitrification, can be neutralized by the denitrification of nitrite. In theory 100% of the ammonium conversion can be performed by the overall reaction of nitrification/denitrification over nitrite, combined with CO_2^- stripping.

Partial nitritation-anammox

From the stoichiometry of the anammox reaction (Eq. 1.10) it can be inferred that anammox bacteria need ammonium and nitrite in a molar ratio around 1:1.3, respectively. To reach this objective, only half of the ammonium needs to be converted in nitrite by AOB in a preceding nitrification step. This is further denoted by the term "partial nitritation". Moreover, the oxidation of nitrite to nitrate carried out by NOB is not necessary.

The global stoichiometry of the total process combining partial nitrification and anammox is represented in equation 1.19.



$$NH_4^+ + 0.85\ O_2 + 1.11\ HCO_3^- \rightarrow 0.44\ N_2 + 0.11\ NO_3^- + 2.56\ H_2O + 1.11\ CO_2 \quad [1.19]$$

In comparison with the conventional nitrification-denitrification process, in the case of the anammox based processes less oxygen is required and no organic matter must be added as external carbon source. The oxygen requirement is only 1.71 g O_2 / g NH_4^+-N.

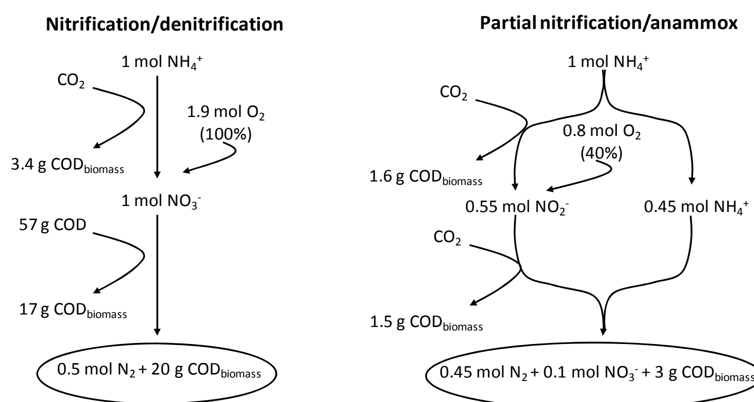


Figure 1.1. Comparison between nitrification-denitrification and partial nitrification-anammox processes to treat wastewaters with low COD/N ratios (Adapted from Fux and Siegrist, 2004).

Two different configurations can be applied in order to combine partial nitritation and anammox processes based in the use of two units or a single one.

Two reactor configuration:

The first unit is operated under aerobic conditions and only half of the ammonium is oxidized to nitrite, the second reactor is operated in anoxic conditions and ammonia and nitrite are converted to nitrogen gas producing a small amount of nitrate.

The most common used system to achieve partial nitritation is the SHARON reactor. It consists of a chemostat reactor operated at temperatures over 30 ºC and operated at a controlled HRT of 1 d to wash out NOB maintaining the AOB inside the reactor. In order to achieve the desired ammonium/nitrite ratio in the effluent the DO concentration and the pH value were the control parameters (Volcke *et al.*, 2006; van Hulle *et al.*, 2007).

Furthermore when the aim is to operate the anammox process at room temperature, other alternatives must be developed to obtain stable partial nitritation, mainly based in oxygen limited biofilm reactors (Garrido *et al.*, 1997; Bernet *et al.*, 2005) and also aerobic granular reactors (Vázquez-Padín *et al.*, 2010b) by keeping the DO concentration in the bulk liquid under certain levels.

One reactor configuration:

The use of a one reactor configuration has been called under different names; CANON: Completely Autotrophic Nitrogen removal Over Nitrite process (Third *et al.*, 2001), OLAND: Oxygen-Limited Autotrophic Nitrification-Denitrification (Kuai and Verstraete, 1998), deammonification (Hippen *et al.*, 1997; Helmer *et al.*, 2001), aerobic deammonification (Wett, 2006) and SNAP: Single-stage Nitrogen removal using anammox and Partial nitritation (Furukawa *et al.*, 2006). The principle is the same in all the cases: both microbial populations, AOB and anammox bacteria, coexist under controlled aerobic conditions.

Under proper oxygen conditions (lower than 0.5% of air saturation) the mixed culture converts ammonia directly into nitrogen gas with nitrite as intermediate product. AOB consume oxygen and generate both nitrite and an anoxic environment for anammox microorganisms.

Two possible strategies to start-up a CANON system are possible: 1) to inoculate an anammox reactor with nitrifying biomass and to supply air to maintain microaerobic conditions (Sliekers *et al.*, 2003) or 2) to operate a nitrifying reactor under oxygen limited conditions to obtain the desired ammonia to nitrite molar ratio inside the system and then to inoculate anammox biomass (Pynaert *et al.*, 2004; Gong *et al.*, 2007; Vázquez-Padín *et al.*, 2009). The second strategy seems to be more suitable due to the observed important

decrease of the anammox activity when the first strategy is applied and in this case only a small amount of anammox biomass is necessary to start-up the CANON process.

Due to the slow growth of both nitrifying and anammox bacteria involved in the CANON process, the use of good biomass retention systems is mandatory to reach significant nitrogen removal rates. In this sense, the development of granular sludge allows the accumulation of large biomass concentrations in the reactors without the need of carrier material addition. Moreover, the use of granular biomass allows the existence of substrate gradients, in such a way that the external layers of the granules can be under aerobic conditions while anoxic conditions are maintained in the core of the aggregates. Therefore, different biological processes can be carried out in the same granule: partial nitrification in the outer layers and anammox in the inner ones (Vlaeminck *et al.*, 2008; Vázquez-Padín *et al.*, 2010a)

1.3. WHERE?: AEROBIC GRANULES

The wastewater biological treatment is often accomplished in the WWTP by means of the application of conventional activated sludge systems. These systems generally require large surface areas for implantation of the treatment and biomass separation units due to the usually poor settling properties of the sludge. The continuous increase of the number of inhabitants, in many cases concentrated in urban areas, provokes the necessity of upgrading already existing WWTPs or building up new ones to cope with the proportional increase of amounts of wastewater production. It is frequent that the space available for these constructions is restricted. This means that the new treatment systems must occupy as less surface as possible. One possible action to reduce the volume of the required units is the development of systems based on the improvement of biomass retention, for example by performing actions to improve the settleability properties of the activated sludge, which allows for the treatment of larger loads. In aerobic systems the growth of biomass adhered to support materials has been widely studied in airlift or fluidised bed reactors (Heijnen *et al.*, 1990; Tijhuis *et al.*, 1996).

In the 90´s of the past century the first studies in aerobic granular sludge were performed. Sludge granulation in aerobic conditions has been firstly reported in continuous operated systems where the removal of the organic matter and/or ammonia nitrogen occurred. Mishima and Nakamura, (1991) used the so called Aerobic Upflow Sludge Blanket (AUSB) to treat a synthetic wastewater previously aerated with pure oxygen to oxidize the organic matter and they obtained granules with diameters ranging from 2 to 8 mm.

Although granular sludge of selected nitrifying sludge is not frequently reported some works are available, such as the initial one of van Benthum *et al.*, (1996) who proposed a dynamic model to explain the formation of nitrifying granules in a Biofilm Airlift Suspension (BAS) reactor through the breaking-up of biofilms formed on basalt particles. Those removed

patches of biofilms grew in form of individual granules. Later Campos *et al.*, (2000) reported the formation of nitrifying granular sludge in an airlift system, fed with a synthetic wastewater, where particle average diameters ranged from 0.22 to 0.36 μm when loads up to 4.1 g NH_4^+-N/(L d) were treated. The common feature of these works was that no control of the formation of the nitrifying granules was achieved and granules were obtained either in special operational conditions in non repeated experiments or using carrier material as initiator.

Aerobic granular biomass is easily developed in Sequencing Batch Reactors (SBR) since these systems fulfil most of the needed requirements for this aim. An increasing number and diversity of studies, reflecting research on aerobic granular sludge, have been developed in the past years. This evolution made it necessary to establish a definition to discern between an aerobic granule and a simple floc with relatively good settling properties. The definition of "aerobic granule" commonly accepted came out from the discussions which took place at the "*1st IWA-Workshop Aerobic Granular Sludge*" in Munich (2004) and it literally stated that:

"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" (de Kreuk *et al.*, 2005).

The explanation of the different parts of the statement was discussed and analyzed during the 2006 workshop and delegates agreed on the statement, when the following conditions are taken into account:

1. "Aggregates of microbial origin": speaking of granular activated sludge in the statement implies that aerobic granules need to contain active microorganisms and cannot only consist of components of microbial origin (as proteins, EPS, etc.). The microbial population in aerobic granular sludge are to be expected more or less similar to the ones in activated sludge or biofilms, thus there is no need to describe specific groups of microorganisms in the definition. Furthermore, this part implies that no carrier material is intentionally involved or added; the aggregate is formed without the dosage of such carrier material.
2. "No coagulation under reduced hydrodynamic shear": this part describes the difference in behaviour between activated sludge and aerobic granular sludge. Activated sludge flocs tend to coagulate when they settle (when liquid-sludge mixture is not aerated or stirred), whilst granules do not coagulate and settle as separate units.
3. "Which settle significantly faster than activated sludge flocs": this means that SVI_{10} (SVI after 10 min of settling) in combination with SVI_{30} should be used for characterising the settleability of granular activated sludge as it was suggested by Schwarzenbeck *et al.*, (2004b). The difference between the SVI_{10} and SVI_{30} value gives an excellent indication about the granule formation and indicates the extent of thickening after settling.

4. The minimum size of the granules should be as such that the biomass still fulfils point three. This minimum size was set to 0.2 mm, which was decided based on measurements in the past. This limit could be adjusted per case/granule type, as long as the other demands of the definition hold.
5. Sieving is considered a proper method to harvest granules from activated sludge tanks or from aerobic granule reactors, which also determines certain strength of the required biomass matrix.

When an aggregate fulfils all characteristics as described above it can be called an aerobic granule. This simplifies the interpretation of experimental results and clarifies the differences between aerobic granular sludge, activated sludge or biofilms.

1.3.1. Formation of granules

Aerobic granulation can be considered 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 operational and environmental conditions have to be fulfilled and some of them are described below.

Feast-Famine regime: Short feeding periods must be selected to create feast and famine stages characterized by the presence or absence of organic matter in the liquid media, respectively. With this feeding strategy the selection of the appropriate microorganisms to form granules is achieved. When the substrate concentration in the bulk liquid is high the granule-former organisms can store the organic matter in form of polyhydroxyalkanoates (PHAs) to be consumed in the famine period, which gives them a competitive advantage compared to the filamentous organisms.

Short settling time: This hydraulic selection pressure on the microbial community allows retaining granular biomass inside the reactor while flocculent one is washed out (Qin *et al.*, 2004).

Hydrodynamic shear force: Evidences show that the application of high shear forces favours the formation of aerobic granules and the physical granule integrity. 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 more compact aerobic granules were developed at high hydrodynamic shear forces (Tay *et al.*, 2001b).

1.4. WHAT FOR?: APPLICATIONS OF AEROBIC GRANULATION

Recent research showed that it is possible to grow granular sludge in Sequencing Batch Reactors (SBR) without carrier material and at large dissolved oxygen concentrations using either a synthetic wastewater (Beun *et al.*, 1999; Dangcong *et al.*, 1999; Beun *et al.*, 2002) or industrial effluents (Arrojo *et al.*, 2004).

1.4.1. Laboratory research in aerobic granular sludge

The interest in applying aerobic granular systems is mainly related to the compactness of its design in comparison to conventional activated sludge wastewater treatment systems. The aerobic granulation in SBRs has been extensively researched in laboratory works to evaluate the effects of settling velocity, size, shape, density, hydrophobicity, physical strength, microbial activity and extracellular polymeric substances (Adav *et al.*, 2008; Campos *et al.*, 2009) on the performance of aerobic granular sludge. However most of these previous studies for the cultivation of aerobic granules were based on the use of synthetic feeding while information associated with urban or industrial wastewater is more limited. Even so, studies performed at laboratory scale showed the potential of the aerobic granular technology to treat wastewaters from industrial and municipal origin (Table 1.1).

Table 1.1. Performance of some aerobic granular reactors with industrial and urban wastewater.

	OLR_{max} (kg COD/ m^3 d)	NLR (kg N/ m^3 d)	COD_{rem} (%)	N_{rem} (%)	SVI (mL/g VSS)	d_{feret} (mm)	Ref.
Dairy products	7.0	0.7	90	70	60	3.5	[1]
Malting	3.2	0.006	80	-	35	-	[2]
Abattoir	2.6	0.35	98	98	22	1.7	[3]
Pharmaceutical industry	5.5	0.03	80	-	-	-	[4]
Dairy plant	5.9	0.28	90	80	50	-	[5]
Soybean-processing	6.0	0.3	98.5	-	26	1.2	[6]
Metal-refinery process	-	1.0	-	95	-	1.1	[7]
Domestic sewage	1.6	0.4	50	11	38	1.1	[8]
Brewery	3.5	0.24	88.7	88.9	32	2-7	[9]
Abattoir	2.7	0.43	85	93	-	0.7-1.6	[10]
Winery	6.0	0.01	95	-	-	2.0	[11]
Municipal	1.0	0.2	90	95	35	0.2-0.8	[12]
Newsprint effluent	-	-	92	-	39	1-2	[13]
Urban + industrial	-	-	80	98	30	0.8	[14]
Seafood	4.0	0.45	90	27	35	2.76	[15]

[1] Arrojo *et al.*, 2004; [2] Schwarzenbeck *et al.*, 2004a; [3] Cassidy and Belia, 2005; [4] Inizan *et al.*, 2005; [5] Schwarzenbeck *et al.*, 2005; [6] Su and Yu, 2005; [7] Tsuneda *et al.*, 2006; [8] de Kreuk and van Loosdrecht, 2006; [9] Wang *et al.*, 2007; [10] Yilmaz *et al.*, 2008; [11] López-Palau *et al.*, 2009; [12] Ni *et al.*, 2009 [13] Liu *et al.*, 2010a]; [14] Liu *et al.*, 2010b [15] Val del Río *et al.*, (accepted)

The use of different types of wastewater indicated that it is possible to obtain aerobic granules with different industrial substrates, and moreover, this technology is suitable to obtain large efficiencies in terms of COD and nutrients (nitrogen and phosphorus) removal. However, a pre- or post-treatment is recommended to fulfil the disposal requirements when important suspended solids concentrations are present (de Bruin *et al.*, 2004).

These results indicated that in aerobic granular reactors organic matter removal efficiencies ranged between 80 and 98%, while the reached nitrogen removal efficiencies, when the system was optimized, were slightly lower (70 – 95%) (Table 1.1). The physical properties of the aerobic granular biomass showed values of SVI lower than 60 mL/g VSS, densities larger than 10 g VSS/$L_{granule}$ and mean average feret diameters (d_{feret}) that ranged between 1.0 and 3.5 mm.

Presence of organic toxic compounds

Research has been also focused on the study of the impact of some toxic pollutants on the performance of aerobic granular sludge due to their special characteristics. Toxic compounds such as phenol, pyridine, chloroanilines, heavy metals, dyes or uranium, that can be found in certain wastewaters from coal and refining, pharmaceutical and pesticide processing industries were tested using synthetic wastewater supplemented with the toxics (Maszenan *et al.*, 2011). Removal mechanisms can include adsorption, absorption and degradation.

Phenol, which is toxic to aquatic species and also bacteria (Allsop *et al.*, 1993; Chung *et al.*, 2003), is biodegradable by activated sludge at low concentrations. Aerobic granules have been applied to degrade phenol and pentachlorophenol (Jiang *et al.*, 2002; Jiang *et al.*, 2004; Tay *et al.*, 2005; Adav and Lee, 2008) and displayed an excellent ability for this purpose. Aerobic granular systems were able to treat an influent concentration of 500 mg phenol/L with the obtaining of a stable effluent phenol concentration of less than 0.2 mg/L (Jiang et al., 2004; Jiang et al., 2002) and with a specific activity of 1.18 g phenol/(g VSS d).

Aerobic granules could efficiently degrade pyridine of initial concentrations of 200 -2500 mg/L. The obtained specific degradation rates of pyridine were 1.75 and 1.58 g pyridine/(g VSS d) at concentrations of 250 and 500 mg/L of pyridine, respectively (Adav *et al.*, 2007).

Chloroanilines are used as important intermediates in the production of synthetic organic chemicals and polymers. Aerobic granules developed in a SBR with synthetic wastewater treated a loading of 0.8 kg/(m^3d) of chloroanilines with a constant removal efficiency of 99.9% (Zhu *et al.*, 2008).

The feasibility of the use of aerobic granules for the biosorption of heavy metals like Zn^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} and Cu^{2+} has also been reported (Liu *et al.*, 2002; Liu *et al.*, 2003a; Liu *et al.*, 2003b; Liu and Xu, 2007; Gai *et al.*, 2008).

Dyes like Malachite Green (MG) (Sun *et al.*, 2006) and Sumifix Black EXA, Sumifix Navy Blue EXF and Synozol Red were also removed by aerobic granules (Muda *et al.*, 2010). The maximum biosorption capacity of Malachite Green onto aerobic granules was of 56.8 mg MG/g of granule.

The use of aerobically grown granular biomass as novel biomaterials for removing soluble uranium has been demonstrated (Nancharaiah *et al.*, 2006). The biosorption of uranium [U(VI)] at different initial pH values, from 1 to 8, and different initial uranium concentrations, from 6 to 750 mg/L, has been studied. Obtained results indicated the occurrence of a rapid biosorption (< 1 h) in the acidic pH range (1 to 6) compared to that at pH 7.0 or above. Moreover, almost complete removal of uranium in the range 6–100 mg/L in less than 1 h was reported.

1.4.2. Pilot research in aerobic granular sludge

The first pilot research project using the aerobic granular technology was built and operated in The Netherlands with the Nereda™ system in order to demonstrate the applicability of the aerobic granular sludge technology for the treatment of municipal wastewater (de Bruin *et al.*, 2005). The reactor was designed for simultaneous organic matter, nitrogen and phosphorous removal using two SBR units with a height of 6 m and a diameter of 0.6 m operated in parallel treating wastewater at a flow rate of 5.0 m^3/h. The formation of aerobic granules took few weeks, and exhibited a SVI of 55 mL/g VSS. In this case, fast formation of granules was observed under conditions of extensive COD removal, extensive biological phosphate removal and low nitrate effluent concentrations (de Bruin *et al.*, 2005).

Other pilot scale reactors using aerobic granular sludge were designed since then all around the world: Netherlands, Portugal, South Africa and China. As an example, a reactor with a volume of 1 m^3 with an internal diameter of 0.5 m and a height of 6 m is installed in China to treat low strength wastewater (Ni *et al.*, 2009)

From the basis of the aerobic granular sludge but using a contention system for the granules, a sequencing batch biofilter granular reactor (SBBGR) with a volume of 3.1 m^3 was developed by IRSA (Istituto di Ricerca Sulle Acque, Italy). Different studies were carried out in this plant treating sewage at an Italian wastewater treatment plant (Di Iaconi *et al.*, 2008; Di Iaconi *et al.*, 2009; Di Iaconi *et al.*, 2010).

With these examples at laboratory and pilot plant scale, it is clear that aerobic granular sludge is a promising alternative for the treatment of wastewater from different sources at real scale. It is remarkable that research was not only focused in the operation of aerobic reactors but also in some aspects like the way to obtain a quick start-up or economical considerations.

In order to reduce the time needed for the start-up of this systems it is possible to apply a similar strategy to that frequently applied in anaerobic reactors: the use of pre-cultured granules seeded into the reactor. Successful start up in a pilot-scale sequencing batch reactor seeded with aerobic granules pre-cultured in a small column reactor has been already achieved (Liu *et al.*, 2005). Moreover, it is possible to store aerobic granules for long periods of time, more than 2 months, and quickly recover their activity in less than 2 weeks (Zhu and Wilderer, 2003; Wang *et al.*, 2008; Pijuan *et al.*, 2009).

Sensitivity analysis performed on the granular SBR technology in comparison with activated sludge indicated that the former is less sensitive to land price, because of its compactness, and it is more sensitive to rain water flow, because of the large impact of the maximum batch volume on the design of the granular SBR. Moreover, this technology is very compact, which is an important advantage in relation to activated sludge technology, especially in densely populated areas. Because of the high allowable volumetric loads, the footprint of the SBR is 75% less compared to the references of conventional activated sludge systems (de Bruin *et al.*, 2004).

Based on total annual costs, granular SBR systems even with pre- or post-treatment proved to be more attractive than the activated sludge alternatives (17% and 6% lower, respectively). However, it is possible that the granular SBR with only pre-treatment could not meet the effluent standards for municipal wastewater treatment because of exceeding the suspended solids effluent standard caused by washout of not well settleable biomass (de Bruin *et al.*, 2004).

1.5. THE MICROBIOLOGY OF WASTEWATER TREATMENT: WHICH?

The performance of a biological wastewater treatment plant strongly depends on the activities and interactions of its microbial community. Thus, information on the identity of microorganisms responsible for specific activities, of interactions between cells of the same or different populations and information on the influence of changing environmental conditions are important for optimizing these processes.

In this context, traditional microbiological techniques and conventional microscopy were not able to answer the many questions about species composition, structure and bacterial distribution as well as the spatial activity (Wagner *et al.*, 1993). To overcome this drawbacks molecular biological techniques, which do not require isolation of bacterial strains, have emerged and nowadays are more and more frequently used to detect and characterize bacteria in natural environments. Among these techniques, cloning and the creation of a gene library, denaturant gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization with DNA probes (FISH) stand out, with a great potential but also some limitations in wastewater treatment systems (Wilderer *et al.*, 2002; Sanz and Köchling, 2007).

1.5.1. Full cycle rRNA approach

The 16S rRNA approach has become the gold standard for the cultivation-independent assessment of bacterial diversity in natural and engineered systems (Amann *et al.*, 1995). The importance of these 16S and 23S rRNA genes is that they code for the rRNA molecules needed for protein synthesis; they are about 1500 and 3000 nucleotides in length, respectively, and contain both highly variable and conserved regions.

The so called full cycle 16S rRNA approach is based on the following steps: the extraction of nucleic acids, their amplification by means of polymerase chain reaction (PCR) and cloning (or DGGE) of the 16S rRNA genes, followed by sequencing and finally identification and affiliation of the isolated clone together with adequate reference sequences with the aid of phylogenetic software. Subsequently, specific oligonucleotide probes for these organisms can be design and used for *in situ* hybridisation of the original sample (FISH) (Figure 1.2).

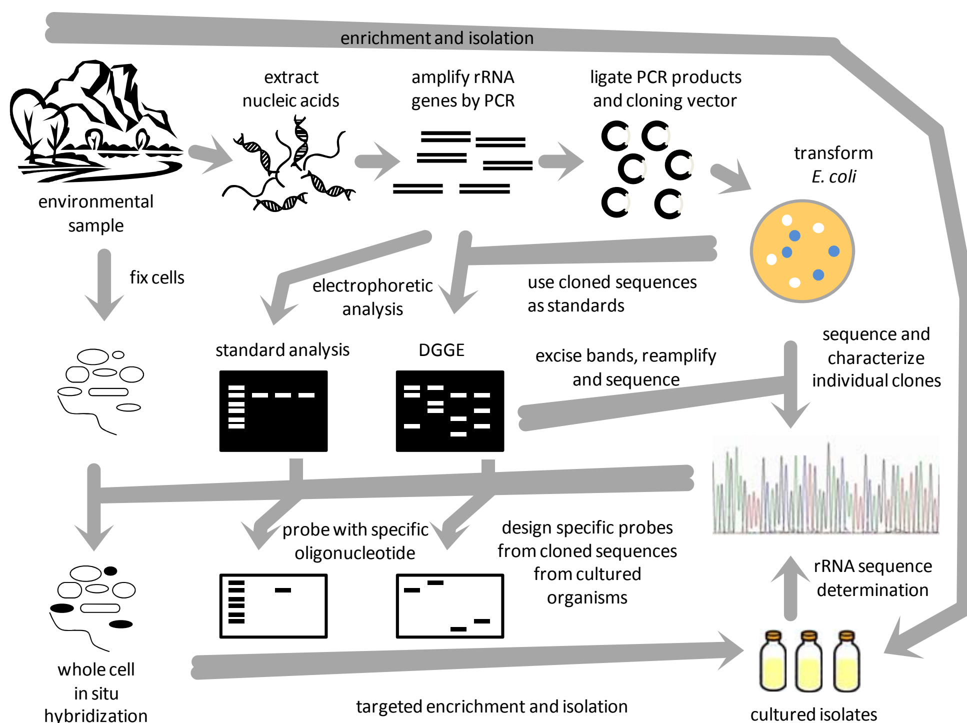


Figure 1.2. Commonly used approaches in molecular microbial ecology (from Head et al., 1998).

Other genes such as the 23S, the 18S (for Eukaryota), the amoA (for ammonia oxidizing bacteria) or HZO (for anaerobic ammonia oxidizing bacteria) have been used. For example, the amoA gene has been used as an additional molecular marker for the detection of ammonia oxidizers (Purkhold *et al.*, 2000; Purkhold *et al.*, 2003) in wastewater treatment

plants. Results obtained with deduced amino acid sequences of the amoA gene fragments showed that are consistent with the 16S rRNA phylogeny of AOB.

By the use of traditional and molecular techniques, many functional groups of bacteria involved in the most common wastewater treatment processes have been identified and described.

1.5.2. Ammonia oxidizing bacteria

Ammonia oxidizing bacteria (AOB) are responsible for the first and rate-limiting step of the nitrification. All known chemolithoautotrophic AOB belong to the phylum *Proteobacteria* (Figure 1.3). Studies dealing with 16S rDNA sequences have classified the AOB into two main monophyletic groups. The first, corresponding to the *Gamma*-subgroup of the *Proteobacteria*, include strains of *Nitrosococcus oceani* and *Nitrosococcus halophilus*. The second is composed by the genera *Nitrosomonas* and *Nitrosospira*, both included into the *Beta*- subgroup of the *Proteobacteria* (Purkhold *et al.*, 2000). The genera *Nitrosolobus* and *Nitrosovibrio* are enclosed within the *Nitrosospira* group (Head *et al.*, 1993) and *Nitrosococcus mobilis* is included within the *Nitrosomonas.*

Although the basic metabolism is more or less uniform for all ammonia-oxidizing bacteria, different physiological requirements exist among the different strains (Wagner *et al.*, 1995; Koops and Pommerening-Roser, 2001). For example, the substrate affinity, salt requirements and salt tolerance differ significantly among ammonia oxidizers. Experiments using lab scale reactors and studies of pure cultures suggested that *N. oligotropha* and closely related AOB are better adapted to low ammonia concentrations than *N. europaea*. *Nitrosococcus mobilis* related to AOB seem to appear especially in reactors treating sludge liquor or other types of wastewater with elevated ammonia and salt concentrations such as those from the food processing wastewater (Juretschko *et al.*, 1998).

In most nitrifying WWTPs, ammonia is oxidized by AOB of the genus *Nitrosomonas* (including *Nitrosococcus mobilis*). AOB of the genus *Nitrosospira* have occasionally been detected in WWTPs, but these AOB are generally more common in terrestrial habitats and seem to play only minor roles for wastewater treatment. In marine habitats, *Nitrosomonas marina*, *Nitrosococcus oceani* and *Nitrosococcus halophilus* are common. Due to their high salt requirements, these AOB are absent from most WWTPs with the possible exception of brackish water and saltwater systems like a marine aquaculture system (Foesel *et al.*, 2008).

In activated sludge flocs and biofilms, AOB related to *Nitrosomonas* usually form almost spherical compact cell aggregates. Single cells within these clusters are well visible. The diameter of most AOB clusters is 10 to 50 μm.

Recently, the existence of ammonia oxidizing bacteria belonging to *Archaea* (AOA) has been discovered. After environmental genomics had provided strong clues that AOA may

occur in soils and oceans, the existence and nitrifying activity of these organisms has been eventually confirmed by isolation of an ammonia-oxidizing archaeon from gravel of a saltwater aquarium (Konneke *et al.*, 2005). Molecular studies have also demonstrated that AOA are more abundant than AOB in different soils and that they are present in extreme environments like hot springs. Although amoA genes have been detected by PCR in treatment plants (Park *et al.*, 2006), it remains unclear whether they play an important role in the nitrification performed in wastewater treatment plants.

1.5.3. Nitrite oxidizing bacteria

Nitrite oxidizing bacteria catalyze the second step of the nitrification process, the oxidation of nitrite to nitrate. This functional group is phylogenetically more heterogeneous than the AOB. All known nitrite oxidizers belong to one of the genera *Nitrobacter*, *Nitrospina*, *Nitrococcus* and *Nitrospira*.

The physiologically best characterized NOB are those members of the genus *Nitrobacter* in the class *Alphaproteobacteria*, which contains four described species that are found in a large range of terrestrial and aquatic habitats and sediments of highly alkaline soda lakes (Sorokin *et al.*, 1998). Each of the genera *Nitrococcus* and *Nitrospina* harbours only one known species. *Nitrococus mobilis* is a member of the *Gammaproteobacteria* whereas *Nitrospina gracilis* belongs to the *Deltaproteobacteria*. *Nitrospira* is part of the phylum *Nitrospirae,* and thus it is not closely related to the other known NOB

Nitrobacter-like bacteria were during long time considered to be the key nitrite oxidizers in biological wastewater treatment. This view was challenged when *Nitrobacter* cells were not detected by FISH with rRNA-targeted oligonucleotide probes in nitrifying activated sludge samples from several treatment plants (Wagner *et al.*, 1996). Subsequently, application of the full-cycle rRNA approach to biomass samples from a full scale industrial treatment plant revealed that novel NOB closely related to the genus *Nitrospira* (and not *Nitrobacter*) which were the predominant nitrite oxidizer in that system (Juretschko *et al.*, 1998). Reactors that temporarily contain elevated nitrite concentrations can reach a relatively high abundance of *Nitrobacter* probably due to the fact that these NOB are adapted to high nitrite concentrations, whereas *Nitrospira* are adapted to low nitrite concentrations (Schramm *et al.*, 1999). The coexistence of both populations is also possible in the same unit (Wagner *et al.*, 2002; Vazquez-Padin *et al.*, 2009).

In the majority of nitrifying WWTPs the dominant NOB are members of the genus *Nitrospira* (Juretschko *et al.*, 1998; Schramm *et al.*, 1998). All known *Nitrospira* in WWTPs form spherical or irregularly shaped cell aggregates, which consist of several hundred or thousand cells. The diameter of these aggregates is 10 to 100 μm, but even larger clusters are occasionally found. *Nitrobacter* usually form cell aggregates like the other nitrifiers, or they can occur as single cells embedded in the biofilm matrix. In flocs and biofilms, NOB often

appears in the direct spatial neighbourhood of AOB, which reflects the mutualistic symbiosis of these two functional groups (Maixner *et al.*, 2006).

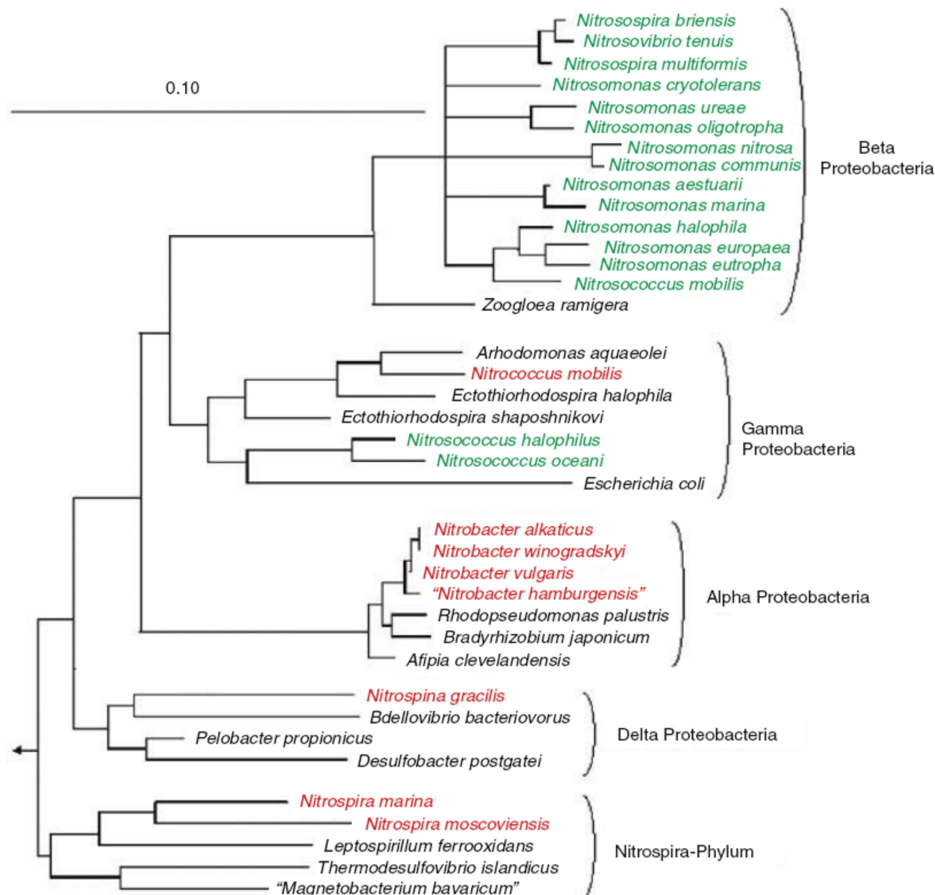


Figure 1.3. 16S rRNA-based tree reflecting the phylogenetic relationship of ammonia- and nitrite-oxidizing bacteria. In the tree, AOB are labeled green, and NOB are depicted in red. Bar represents 10% estimated sequence divergence (from Bock and Wagner, 2006).

1.5.4. Anammox bacteria

The attempts to isolate the anammox organism from enrichment cultures were unsuccessful. Eventually, culture independent molecular methods revealed that these novel microbes were members of a deep-branching lineage in the bacterial phylum *Planctomycetes* (Strous *et al.*, 1999a). They are peculiar prokaryotes by the following characteristics: they propagate by budding instead of binary fission, and posses an intracellular compartment called "anammoxosome" (Lindsay *et al.*, 2001). In addition they have an unusual cell envelope structure and contain ladderanes (Damste *et al.*, 2002).

Since the discovery of anammox bacteria, several different representatives have been enriched from WWTPs. These are the *Candidatus* lineages *Kuenenia* with "K. stuttgartiensis"(Schmid *et al.*, 2000), *Brocadia* with the species "B. anammoxidans" (Strous *et al.*, 1999a) and "B. fulgida" (Kartal *et al.*, 2008) and *Anammoxoglobus* with "A. propionicus" (Kartal *et al.*, 2007). One important fact is that some anammox bacteria were enriched using a medium containing propionate ("A. propionicus") or acetate ("B. fulgida") and could co-oxidize organic compounds such as formate, propionate, monomethylamine and dimethylamine at high rate and in this way out-compete the other anammox species for nitrite.

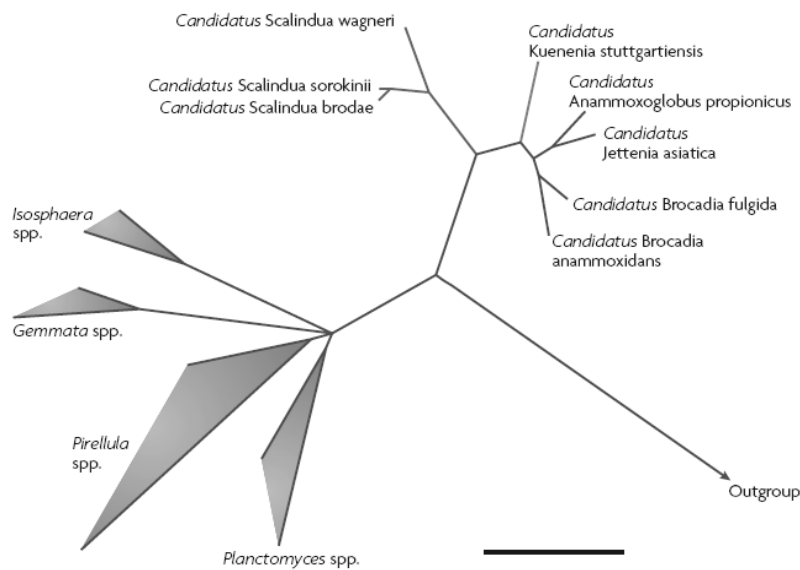


Figure 1.4. 16s rRNA gene-based phylogenetic tree of anammox bacteria. Illustrates the relationships of the different families of anaerobic ammonium oxidation (anammox) bacteria among the *Planctomycetes*. The scale bar represents 10% sequence divergence (Kuenen, 2008).

Up to date the studies about the enrichment or operation of the anammox process at laboratory or pilot scale reactors were performed mainly with bacteria of the genus *C.* "Brocadia" or *C.* "Kuenenia" (van der Star *et al.*, 2007), so the major part of the available information on anammox is about these types of bacteria.

Usually, anammox bacteria occur as small to large cell aggregates. Anammox have coccoid morphology and a "donut-like" shape after the application of the FISH technique with an apparent small hole in the centre of the cells caused by the absence of ribosomes.

1.5.5. Denitrifying bacteria

Denitrifying ability is distributed in a wide variety of bacterial groups; most of them being facultative. However, the capacity to denitrify has also been found in some archaea and in fungi (Oishi and Kusuda, 2003; Cabello *et al.*, 2004). The denitrifying bacteria are facultative anaerobic heterotrophs and, less frequently autotrophs. Under oxic conditions most denitrifiers carry out full aerobic respiration. These facultative denitrifiers seem to constitute a major fraction of the whole of bacteria present in the activated sludge community (Nielsen and Nielsen, 2002). The organisms capable of performing the denitrification process are not limited to specific microbial taxa but they are also present in many phylogenetic subgroups. Previously culture dependent studies of denitrifiers typically described these organisms to be members of *Pseudomonas*, *Paracoccus*, *Rhizobium*, *Bacillus* or *Alcaligenes*, but these are usually not the dominant genera in the activated sludge process. Several publications based on both culture dependent and culture independent approaches indicate that other denitrifiers must be the dominant ones, therefore, denitrifiers are found among the *Proteobacteria*, *Firmicutes* and the *Bacteroidetes*, covering more than 50 genera.

Studies carried out directly in a WWTP have shown that *Betaproteobacteria* have a great importance. Within the *Comamonadaceae* family bacteria belonging to the genera *Curvibacter* and *Acidovorax* and within the *Rhodocyclaceae* family, belonging to the genera *Zoogloea*, *Azoarcus* and *Thauera* were found as dominating denitrifiers in different WWTPs (Juretschko *et al.*, 2002; Wagner and Loy, 2002; Thomsen *et al.*, 2004; Thomsen *et al.*, 2007). In WWTPs with Enhanced Biological Phosphorus Removal (EBPR) combined with nitrogen removal, polyphosphate accumulating members of *Accumulibacter* (*Betaproteobacteria*) were present and some of these, but presumably not all, were capable of performing the denitrification (Kong *et al.*, 2004). Studies carried out in a denitrifying lab scale reactor inoculated with activated sludge and run on acetate revealed high diversities of potential denitrifying organisms belonging to *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Actinobacteria* (Ginige *et al.*, 2004).

Nowadays the performance of the biological wastewater treatments has still certain uncertainties which implies the necessity to explore new solutions from the point of view of the development of new systems and the ways to the better understand the biological processes and the organisms involved.

1.6. REFERENCES

Adav S. and Lee D.J. (2008). Physiological characterization and interactions of isolates in phenol-degrading aerobic granules. *Applied Microbiology and Biotechnology,* 78(5), 899-905.

Adav S.S., Lee D.J. and Ren N.Q. (2007). Biodegradation of pyridine using aerobic granules in the presence of phenol. *Water Research,* 41(13), 2903-2910.

Adav S.S., Lee D.J., Show K.Y. and Tay J.H. (2008). Aerobic granular sludge: Recent advances. *Biotechnology Advances,* 26(5), 411-423.

Allsop P.J., Chisti Y., Mooyoung M. and Sullivan G.R. (1993). Dynamics of phenol degradation by *Pseudomonas putida*. *Biotechnology and Bioengineering,* 41(5), 572-580.

Amann R., Ludwig W. and Schleifer K.H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews,* 59(1), 143-169.

Anthonisen A.C., Loehr R.C., Prakasam T.B.S. and Srinath E.G. (1976). Inhibition of nitrification by ammonia and nitrous acid. *Journal Water Pollution Control Federation,* 48(5), 835-852.

Arrojo B., Mosquera-Corral A., Garrido J.M. and Mendez R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research,* 38(14-15), 3389-3399.

Bernet N., Sanchez O., Cesbron D., Steyer J.P. and Delgenès J.P. (2005). Modeling and control of nitrite accumulation in a nitrifying biofilm reactor. *Biochemical Engineering Journal,* 24(2), 173-183.

Bettazzi E., Caffaz S., Vannini C. and Lubello C. (2010). Nitrite inhibition and intermediates effects on Anammox bacteria: A batch-scale experimental study. *Process Biochemistry,* 45(4), 573-580.

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(3), 702-712.

Bock E. and Wagner M. (2006). Oxidation of inorganic nitrogen compounds as an energy source in *The Prokaryotes: Ecophysiology and Biochemistry, vol. 2*. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. (eds), Springer, New York.

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

Cabello P., Roldan M.D. and Moreno-Vivian C. (2004). Nitrate reduction and the nitrogen cycle in archaea. *Microbiology,* 150(11), 3527-3546.

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

Campos J.L., Figueroa M., Mosquera-Corral A. and Mendez R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering,* 1, 136-151.

Carrera J., Jubany I., Carvallo L., Chamy R. and Lafuente J. (2004). Kinetic models for nitrification inhibition by ammonium and nitrite in a suspended and an immobilised biomass systems. *Process Biochemistry,* 39(9), 1159-1165.

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(19), 4817-4823.

Chung T.P., Tseng H.Y. and Juang R.S. (2003). Mass transfer effect and intermediate detection for phenol degradation in immobilized Pseudomonas putida systems. *Process Biochemistry,* 38(10), 1497-1507.

Damste J.S.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(6908), 708-712.

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

Dapena-Mora A., Fernandez I., Campos J.L., Mosquera-Corral A., Mendez R. and Jetten M.S.M. (2007). Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology,* 40(4), 859-865.

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: an alternative to activated sludge? *Water Science and Technology,* 49(11-12), 1-7.

de Bruin L.M.M., van der Roest H.F.R., de Kreuk M., van Loosdrecht M.C.M., Bathe S., McSwain B.S. and Schwarzenbeck N. (2005). Promising results pilot research aerobic granular sludge technology at WWTP Ede in *Aerobic Granular Sludge*. pp. 135-142, IWA Publishing, London.

de Kreuk M.K., McSwain B.S., Bathe S., Tay S.T.L., Schwarzenbeck N. and Wilderer P.A. (2005). Discussion outcomes in *Aerobic granular sludge*. Bathe, S., de Kreuk, M., Mc Swain, B.S. and Schwarzenbeck, N. (eds), pp. 165-169, IWA, London.

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

Di Iaconi C., De Sanctis M., Rossetti S. and Ramadori R. (2008). Technological transfer to demonstrative scale of sequencing batch biofilter granular reactor (SBBGR) technology for municipal and industrial wastewater treatment. *Water Science and Technology,* 58(2), 367-372.

Di Iaconi C., Moro G.D., Pagano M. and Ramadori R. (2009). Municipal landfill leachate treatment by SBBGR technology. *International Journal of Environment and Waste Management,* 4, 422-432.

Di Iaconi C., De Sanctis M., Rossetti S. and Ramadori R. (2010). SBBGR technology for minimising excess sludge production in biological processes. *Water Research,* 44(6), 1825-1832.

Fernandez I., Vazquez-Padin J.R., Mosquera-Corral A., Campos J.L. and Mendez R. (2008). Biofilm and granular systems to improve Anammox biomass retention. *Biochemical Engineering Journal,* 42(3), 308-313.

Fernández I., Dosta J., Fajardo C., Campos J.L., Mosquera-Corral A. and Méndez R. Short- and long-term effects of ammonium and nitrite on the Anammox process. *Journal of Environmental Management,* In Press, Corrected Proof. doi: 10.1016/j.jenvman.2010.10.044

Foesel B.U., Gieseke A., Schwermer C., Stief P., Koch L., Cytryn E., De La Torré J.R., Van Rijn J., Minz D., Drake H.L. and Schramm A. (2008). Nitrosomonas Nm143-like ammonia oxidizers and Nitrospira marina-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. *FEMS Microbiology Ecology,* 63(2), 192-204.

Furukawa K., Lieu P.K., Tokitoh H. and Fujii T. (2006). Development of single-stage nitrogen removal using anammox and partial nitritation (SNAP) and its treatment performances. *Water Science and Technology,* 53(6), 83-90.

Fux C. and Siegrist H. (2004). Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: environmental and economical considerations. *Water Science and Technology,* 50(10), 19-26.

Gai L.H., Wang S.G., Gong W.X., Liu X.W., Gao B.Y. and Zhang H.Y. (2008). Influence of pH and ionic strength on Cu(II) biosorption by aerobic granular sludge and biosorption mechanism. *Journal of Chemical Technology and Biotechnology,* 83(6), 806-813.

Garrido J.M., van Benthum W.A.J., van Loosdrecht M.C.M. and Heijnen J.J. (1997). Influence of dissolved oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. *Biotechnology and Bioengineering,* 53(2), 168-178.

Ginige M.P., Hugenholtz P., Daims H., Wagner M., Keller J. and Blackall L.L. (2004). Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. *Applied and Environmental Microbiology,* 70(1), 588-596.

Gong Z., Yang F., Liu S., Bao H., Hu S. and Furukawa K. (2007). Feasibility of a membrane-aerated biofilm reactor to achieve single-stage autotrophic nitrogen removal based on Anammox. *Chemosphere,* 69(5), 776-784.

Grady C.P.L., Daigger G.T. and Lim H.C. (1999) Biological wastewater treatment, Marcel Dekker, Inc., New York.

Head I.M., Hiorns W.D., Embley T.M., McCarthy A.J. and Saunders J.R. (1993). The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. *Journal of General Microbiology,* 139(6), 1147-1153.

Head I.M., Saunders J.R. and Pickup R.W. (1998). Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology,* 35(1), 1-21.

Heijnen S.J., Mulder A., Weltevrede R., Hols 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(1), 202-208.

Helmer C., Tromm C., Hippen A., Rosenwinkel K.H., Seyfried C.F. and Kunst S. (2001). Single stage biological nitrogen removal by nitritation and anaerobic ammonium oxidation in biofilm systems. *Water Science and Technology,* 43(1), 311-320.

Hellinga C., Schellen A.A.J.C., Mulder J.W., van Loosdrecht M.C.M. and Heijnen J.J. (1998). The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. *Water Science and Technology,* 37(9), 135-142.

Hippen A., Rosenwinkel K.H., Baumgarten G. and Seyfried C.F. (1997). Aerobic deammonification: A new experience in the treatment of wastewaters. *Water Science and Technology,* 35(10), 111-120.

Inizan M., Freval A., Cigana J. and Meinhold J. (2005). Aerobic granulation in a sequencing batch reactor (SBR) for industrial wastewater treatment. *Water Science and Technology,* 52(10-11), 335-343.

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,* 35(5), 439-445.

Jiang H.L., Tay J.H., Maszenan A.M. and Tay S.T.L. (2004). Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation. *Applied and Environmental Microbiology,* 70(11), 6767-6775.

Juretschko S., Timmermann G., Schmid M., Schleifer K.H., Pommerening-Roser A., Koops H.P. and Wagner M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. *Applied and Environmental Microbiology,* 64(8), 3042-3051.

Juretschko S., Loy A., Lehner A. and Wagner M. (2002). The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Systematic and Applied Microbiology,* 25(1), 84-99.

Kartal B., Rattray J., van Niftrik L.A., van de Vossenberg J., Schmid M.C., Webb R.I., Schouten S., Fuerst J.A., Damste J.S., Jetten M.S.M. and Strous M. (2007). Candidatus "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology,* 30(1), 39-49.

Kartal B., van Niftrik L., Rattray J., van de Vossenberg J.L.C.M., Schmid M.C., Sinninghe Damste J., Jetten M.S.M. and Strous M. (2008). Candidatus "Brocadia fulgida": an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiology Ecology,* 63(1), 46-55.

Kimura Y., Isaka K., Kazama F. and Sumino T. (2010). Effects of nitrite inhibition on anaerobic ammonium oxidation. *Applied Microbiology and Biotechnology,* 86(1), 359-365.

Kong Y., Nielsen J.L. and Nielsen P.H. (2004). Microautoradiographic study of rhodocyclus-related polyphosphate-accumulating bacteria in full-scale enhanced biological phosphorus removal plants. *Applied and Environmental Microbiology,* 70(9), 5383-5390.

Konneke M., Bernhard A.E., de la Torre J.R., Walker C.B., Waterbury J.B. and Stahl D.A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature,* 437(7058), 543-546.

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

Kuai L. and Verstraete W. (1998). Ammonium removal by the Oxygen-Limited Autotrophic Nitrification-Denitrification system. *Applied and Environmental Microbiology,* 64(11), 4500-4506.

Kuenen J.G. (2008). Anammox bacteria: from discovery to application. *Nature Reviews Microbiology,* 6(4), 320-326.

Lindsay M.R., Webb R.I., Strous M., Jetten M.S., Butler M.K., Forde R.J. and Fuerst J.A. (2001). Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. *Archives of Microbiology,* 175(6), 413-429.

Liu J.R., Nguyen D. and Paice M. (2010a). Aerobic granule formation in a sequencing batch reactor treating newsprint effluent under low phosphate conditions. *Water Science and Technology,* 62(11), 2571-2578.

Liu Q.S., Liu Y., Tay S.T.L., Show K.Y., Ivanov V., Benjamin M. and Tay J.H. (2005). Startup of pilot-scale aerobic granular sludge reactor by stored granules. *Environmental Technology,* 26(12), 1363-1369.

Liu Y., Yang S.F., Tan S.F., Lin Y.M. and Tay J.H. (2002). Aerobic granules: a novel zinc biosorbent. *Letters in Applied Microbiology,* 35(6), 548-551.

Liu Y., Xu H., Yang S.F. and Tay J.H. (2003a). A general model for biosorption of Cd2+, Cu2+ and Zn2+ by aerobic granules. *Journal of Biotechnology,* 102(3), 233-239.

Liu Y., Yang S.F., Xu H., Woon K.H., Lin Y.M. and Tay J.H. (2003b). Biosorption kinetics of cadmium(II) on aerobic granular sludge. *Process Biochemistry,* 38(7), 997-1001.

Liu Y. and Xu H. (2007). Equilibrium, thermodynamics and mechanisms of Ni2+ biosorption by aerobic granules. *Biochemical Engineering Journal,* 35(2), 174-182.

Liu Y.Q., Moy B., Kong Y.H. and Tay J.H. (2010b). Formation, physical characteristics and microbial community structure of aerobic granules in a pilot-scale sequencing batch reactor for real wastewater treatment. *Enzyme and Microbial Technology,* 46(6), 520-525.

López-Palau S., Dosta J. and Mata-Álvarez J. (2009). Start-up of an aerobic granular sequencing batch reactor for the treatment of winery wastewater. *Water Science and Technology,* 60(4), 1049-1054.

Maixner F., Noguera D.R., Anneser B., Stoecker K., Wegl G., Wagner M. and Daims H. (2006). Nitrite concentration influences the population structure of Nitrospira-like bacteria. *Environmental Microbiology,* 8(8), 1487-1495.

Maszenan A.M., Liu Y. and Ng W.J. (2011). Bioremediation of wastewaters with recalcitrant organic compounds and metals by aerobic granules. *Biotechnology Advances,* 29(1), 111-123.

Mishima K. and Nakamura M. (1991). Self-immobilization of aerobic activated sludge - A pilot study of the Aerobic Upflow Sludge Blanket process in municipal sewage treatment. *Water Science and Technology,* 23(4-6), 981-990.

Muda K., Aris A., Salim M.R., Ibrahim Z., Yahya A., van Loosdrecht M.C.M., Ahmad A. and Nawahwi M.Z. (2010). Development of granular sludge for textile wastewater treatment. *Water Research,* 44(15), 4341-4350.

Mulder A., Vandegraaf A.A., Robertson L.A. and Kuenen J.G. (1995). Anaerobic Ammonium Oxidation Discovered in a Denitrifying Fluidized-Bed Reactor. *FEMS Microbiology Ecology,* 16(3), 177-183.

Nancharaiah Y.V., Joshi H.M., Mohan T.V.K., Venugopalan V.P. and Narasimhan S.V. (2006). Aerobic granular biomass: a novel biomaterial for efficient uranium removal. *Current Science,* 91(4), 503-509.

Ni B.-J., Xie W.-M., Liu S.-G., Yu H.-Q., Wang Y.-Z., Wang G. and Dai X.-L. (2009). Granulation of activated sludge in a pilot-scale sequencing batch reactor for the treatment of low-strength municipal wastewater. *Water Research,* 43(3), 751-761.

Nielsen J.L. and Nielsen P.H. (2002). Enumeration of acetate-consuming bacteria by microautoradiography under oxygen and nitrate respiring conditions in activated sludge. *Water Research,* 36(2), 421-428.

Oishi K. and Kusuda T. (2003). Characteristics and distributions of nitrous oxide-producing denitrifying fungi in natural environments. *Journal of Water and Environment Technology,* 1(1), 67-72.

Park H.-D., Wells G.F., Bae H., Criddle C.S. and Francis C.A. (2006). Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Applied and Environmental Microbiology,* 72(8), 5643-5647.

Pijuan M., Werner U. and Yuan Z. (2009). Effect of long term anaerobic and intermittent anaerobic/aerobic starvation on aerobic granules. *Water Research,* 43(14), 3622-3632.

Purkhold U., Pommerening-Roser A., Juretschko S., Schmid M.C., Koops H.P. and Wagner M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. *Applied and Environmental Microbiology,* 66(12), 5368-5382.

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

Pynaert K., Smets B.F., Beheydt D. and Verstraete W. (2004). Start-up of autotrophic nitrogen removal reactors via sequential biocatalyst addition. *Environmental Science & Technology,* 38(4), 1228-1235.

Qin L., Liu Y. and Tay J.H. (2004). Effect of settling time on aerobic granulation in sequencing batch reactor. *Biochemical Engineering Journal,* 21(1), 47-52.

Sanz J.L. and Köchling T. (2007). Molecular biology techniques used in wastewater treatment: An overview. *Process Biochemistry,* 42(2), 119-133.

Schmid M., Twachtmann U., Klein M., Strous M., Juretschko S., Jetten M., Metzger J.W., Schleifer K.H. and Wagner M. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Systematic and Applied Microbiology,* 23(1), 93-106.

Schramm A., de Beer D., Wagner M. and Amann R. (1998). Identification and activities in situ of Nitrosospira and Nitrospira spp. as dominant populations in a nitrifying fluidized bed reactor. *Applied and Environmental Microbiology,* 64(9), 3480-3485.

Schramm A., de Beer D., van den Heuvel J.C., Ottengraf S. and Amann R. (1999). Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. *Applied and Environmental Microbiology,* 65(8), 3690-3696.

Schwarzenbeck N., Erley R., Mc Swain B.S., Wilderer P.A. and Irvine R.L. (2004a). Treatment of malting wastewater in a granular sludge sequencing batch reactor (SBR). *Acta Hydrochimica et Hydrobiologica,* 32(1), 16-24.

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

Schwarzenbeck N., Borges J.M. and Wilderer P.A. (2005). Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor. *Applied Microbiology and Biotechnology,* 66(6), 711-718.

Sliekers A.O., 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(2), 339-344.

Sorokin D.Y., Muyzer G., Brinkhoff T., Kuenen J.G. and Jetten M.S.M. (1998). Isolation and characterization of a novel facultatively alkaliphilic Nitrobacter species, N-alkalicus sp. nov. *Archives of Microbiology,* 170(5), 345-352.

Strous M., vanGerven E., Kuenen J.G. and Jetten M. (1997). Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge. *Applied and Environmental Microbiology,* 63(6), 2446-2448.

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(5), 589-596.

Strous M., Fuerst J.A., Kramer E.H.M., Logemann S., Muyzer G., van de Pas-Schoonen K.T., Webb R., Kuenen J.G. and Jetten M.S.M. (1999a). Missing lithotroph identified as new planctomycete. *Nature,* 400(6743), 446-449.

Strous M., Kuenen J.G. and Jetten M.S.M. (1999b). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology,* 65(7), 3248-3250.

Strous M., Kuenen J.G., Fuerst J.A., Wagner M. and Jetten M.S.M. (2002). The anammox case - A new experimental manifesto for microbiological eco-physiology. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 81(1-4), 693-702.

Su K.Z. and Yu H.Q. (2005). Formation and characterization of aerobic granules in a sequencing batch reactor treating soybean-processing wastewater. *Environmental Science & Technology,* 39(8), 2818-2827.

Sun F.Y., Yang C.Y., Li J.Y. and Yang Y.J. (2006). Influence of different substrates on the formation and characteristics of aerobic granules in sequencing batch reactors. *Journal of Environmental Sciences,* 18 (5), 864-871.

Tay J.H., Liu Q.S. and Liu Y. (2001a). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *Journal of Applied Microbiology,* 91(1), 168-175.

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(1-2), 227-233.

Tay S.T.L., Moy B.Y.P., Jiang H.L. and Tay J.H. (2005). Rapid cultivation of stable aerobic phenol-degrading granules using acetate-fed granules as microbial seed. *Journal of Biotechnology,* 115(4), 387-395.

Tchobanoglous G., Burton F.L. and Stensel H.D. (2003) Wastewater engineering: treatment and reuse, Metcalf & Eddy, Inc.

Third K.A., Sliekers A.O., Kuenen J.G. and Jetten M.S.M. (2001). The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under Ammonium Limitation: Interaction and Competition between Three Groups of Bacteria. *Systematic and Applied Microbiology,* 24(4), 588-596.

Thomsen T.R., Nielsen J.L., Ramsing N.B. and Nielsen P.H. (2004). Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. *Environmental Microbiology,* 6(5), 470-479.

Thomsen T.R., Kong Y. and Nielsen P.H. (2007). Ecophysiology of abundant denitrifying bacteria in activated sludge. *FEMS Microbiology Ecology,* 60(3), 370-382.

Tijhuis L., Hijman B., Vanloosdrecht 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(1-2), 7-17.

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.

Val del Río A., Figueroa M., Mosquera-Corral A., Campos J.L. and Mendez R. Aerobic granulation with effluent of seafood industry *In preparation*.

van Benthum W.A.J., GarridoFernandez J.M., Tijhuis L., vanLoosdrecht 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(6), 764-772.

van der Star W.R.L., Abma W.R., Blommers D., Mulder J.W., Tokutomi T., Strous M., Picioreanu C. and van Loosdrecht M.C.M. (2007). Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Water Research,* 41(18), 4149-4163.

van Hulle S.W.H., Volcke E.I.P., Teruel J.L., Donckels B., van Loosdrecht M.C.M. and Vanrolleghem P.A. (2007). Influence of temperature and pH on the kinetics of the Sharon nitritation process. *Journal of Chemical Technology and Biotechnology,* 82(5), 471-480.

Vázquez-Padín J., Mosquera-Corral A., Campos J.L., Méndez R. and Revsbech N.P. (2010a). Microbial community distribution and activity dynamics of granular biomass in a CANON reactor. *Water Research,* 44(15), 4359-4370.

Vazquez-Padin J.R., Figueroa M., Mosquera-Corral A., Campos J.L. and Mendez R. (2009). Population dynamics of nitrite oxidizers in nitrifying granules. *Water Science and Technology,* 60(10), 2529-2536.

Vázquez-Padín J.R., Pozo M.J., Jarpa M., Figueroa M., Franco A., Mosquera-Corral A., Campos J.L. and Méndez R. (2009). Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR. *Journal of Hazardous Materials,* 166(1), 336-341.

Vázquez-Padín J.R., Figueroa M., Campos J.L., Mosquera-Corral A. and Méndez R. (2010b). Nitrifying granular systems: A suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology,* 74(2), 178-186.

Vlaeminck S.E., Cloetens L.F.F., Carballa M., Boon N. and Verstraete W. (2008). Granular biomass capable of partial nitritation and anammox. *Water Science and Technology,* 58(5), 1113-1120.

Volcke E.I.P., van Loosdrecht M.C.M. and Vanrolleghem P.A. (2006). Controlling the nitrite : ammonium ratio in a SHARON reactor in view of its coupling with an Anammox process. *Water Science and Technology,* 53(4-5), 45-54.

Wagner M., Amann R., Lemmer H. and Schleifer K.H. (1993). Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for

describing microbial community structure. *Applied and Environmental Microbiology,* 59(5), 1520-1525.

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(2), 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(1-2), 237-244.

Wagner M. and Loy A. (2002). Bacterial community composition and function in sewage treatment systems. *Current Opinion in Biotechnology,* 13(3), 218-227.

Wagner M., Loy A., Nogueira R., Purkhold U., Lee N. and Daims H. (2002). Microbial community composition and function in wastewater treatment plants. *Antonie van Leeuwenhoek,* 81(1-4), 665-680.

Wang S.G., Liu X.W., Gong W.X., Gao B.Y., Zhang D.H. and Yu H.Q. (2007). Aerobic granulation with brewery wastewater in a sequencing batch reactor. *Bioresource Technology,* 98(11), 2142-2147.

Wang X.H., Zhang H.M., Yang F.L., Wang Y.F. and Gao M.M. (2008). Long-term storage and subsequent reactivation of aerobic granules. *Bioresource Technology,* 99(17), 8304-8309.

Wett B. (2006). Solved upscaling problems for implementing deammonification of rejection water. *Water Science and Technology,* 53(12), 121-128.

Wiesmann U. (1994). Biological nitrogen removal from wastewater in *Advances in Biochemical Engineering/Biotechnology*. pp. 113-154, Heidelberg.

Wilderer P.A., Bungartz H.J., Lemmer H., Wagner M., Keller J. and Wuertz S. (2002). Modern scientific methods and their potential in wastewater science and technology. *Water Research,* 36(2), 370-393.

Yilmaz G., Lemaire R., Keller J. and Yuan Z. (2008). Simultaneous nitrification, denitrification, and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. *Biotechnology and Bioengineering,* 100(3), 529-541.

Zhu J.R. and Wilderer P.A. (2003). Effect of extended idle conditions on structure and activity of granular activated sludge. *Water Research,* 37(9), 2013-2018.

Zhu L., Xu X., Luo W., Cao D. and Yang Y. (2008). Formation and microbial community analysis of chloroanilines-degrading aerobic granules in the sequencing airlift bioreactor. *Journal of Applied Microbiology,* 104(1), 152-160.

Chapter 2:

Materials and Methods

Summary

In this chapter, the analytical methods used in this work are described. They comprise the conventional parameters used for the wastewater (organic matter, nitrogenous compounds, pH, dissolved oxygen, solids and carbon compounds concentrations) and the biomass characterisation present in the different experimental set-up.

From the conventional chemical parameters measured in the liquid or solid phase, e.g. the Volatile Suspended Solids (VSS), that were determined following Standard Methods (APHA-AWWA-WPCF, 2005) are not described in this chapter. Total Organic and Inorganic Carbon (TOC, IC) and several inorganic ions (NH_4^+, NO_2^-, NO_3^-, Cl^-, PO_4^{3-} and SO_4^{2-}) have been measured by automated systems, and together with other parameters such as sludge volume index (SVI), granules density and techniques of digital image analysis that were optimised in our laboratory are thus described in detail throughout this chapter.

Finally, molecular techniques have been applied to the study of the microbial populations involved in the biological processes and are also described. Among these techniques, cloning and the creation of a gene library, denaturant gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (FISH) with oligonucleotide probes.

2.1. ANALYSIS OF THE LIQUID PHASE

In this section, the methods used for the determination of the conventional parameters to characterize the wastewater composition are described. For the analysis of the soluble fraction, the samples were previously filtered with a pore size filter of 0.45 µm (MF-Millipore, Millipore) in order to remove the suspended solids.

2.1.1. From the *Standard Methods*

The following compounds were determined following the methods reported in *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005):

- Ammonia concentration was determined following the method 4500-NH_3 F. (Phenate method).
- Nitrite concentration was determined following the method 4500-NO_2^- B (Colorimetric Method).
- Nitrate concentration was determined following the method 4500-NO_3^- B (Ultraviolet Spectrophotometric Screening Method)

2.1.2. 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 (in the present case the 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 equivalents.

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* (APHA-AWWA-WPCF, 2005). The total COD (COD_t) is determined using the raw sample.

Reagents preparation

Digestion solution of potassium dichromate: 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.

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.

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

Potassium dichromate solution (0.05 M): 1.226 g of $K_2Cr_2O_7$, previously dried at 105 °C for 2 hours, are dissolved in 500 mL of distilled water.

Ferrous ammonium sulphate titrant (FAS) (0.035 N): 13.72 g of $Fe(NH)_4(SO)_2 \cdot 6H2O$ 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 Pyrex® glass tubes. Add 1.5 mL of digestion solution and 3.5 mL of sulphuric acid reagent slowly on the wall of the Pyrex® tube slightly inclined (to avoid mixing). A blank sample using distilled water is prepared in the same way. This blank acts as "reference", corresponding to the COD contain of the distilled water. After being sealed with Teflon and tightly capped, the Pyrex® tubes are finally mixed completely and placed in the block digester (16500-100, HACH) preheated to 150 °C. The duration of the digestion period is 2 h. After digestion, the Pyrex® tubes are cooled to room temperature. Then, the content of the tubes is transferred to a beaker and, after addition of 1-2 drops of ferroin indicator; the solution is titrated under rapid stirring with the FAS. The FAS solution is standardised daily as follows: 5 mL of distilled water are located into a small beaker, 3.5 mL of sulphuric acid reagent are added. The mixture is cooled to room temperature and 5 mL of potassium dichromate solution (0.05 N) are added. To finish 1-2 drops of ferroin indicator are added and this mixture is titrated with FAS titrant. The end-point corresponds to a colour change from blue-green to reddish brown. Molarity of the FAS solution and COD concentration of the samples are calculated with the equations [2.1] and [2.2]:

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

$$COD = \frac{(A - B) \times M_{FAS} \times 8000}{V} \quad [2.2]$$

Where

M_{FAS}: molarity of the FAS solution (mol/L)

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

COD: Chemical Oxygen Demand concentration (mg O_2/L)

A: mL of FAS solution consumed by the blank

B: mL of FAS solution consumed by the sample

8000: milliequivalent weight of oxygen x 1000 mL/L.

2.1.3. 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 it 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, hydrogen and inorganics that can contribute to the oxygen demand measured by COD (APHA-AWWA-WPCF, 2005). To determine the quantity of organically bound carbon, the organic molecules must be broken down and converted to a single carbon molecular form that can be measured quantitatively. The TOC concentration was determined by a Shimadzu analyzer (TOC-5000) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. The instrument is connected to an automated sampler (Shimadzu, ASI-5000-S). The TC concentrations are determined from the amount of CO_2 produced during the combustion of the sample at 680 °C, using platinum immobilised over alumina spheres as catalyst. The IC concentrations are obtained from the CO_2 produced in the chemical decomposition of the sample with H_3PO_4 (25%) at room temperature. The CO_2 produced is optically measured with a nondispersive infrarred analyzer after being cooled and dried. High purity air is used as carrier gas with a flow of 150 mL/min. A curve comprising four calibration points in the range of 0 to 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.

2.1.4. Inorganic ions

The anions nitrite (NO_2^-), nitrate (NO_3^-), chloride (Cl^-), bromide (Br^-), phosphate (PO_4^-) sulphate (SO_4^-), thiosulphate ($S_2O_3^{-2}$) and the cations lithium (Li^+), sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) were determined by ion chromatography (IC) with a Advanced Compact IC system (861, Metrohm) equipped with a CO_2 suppressor (MCS 853, Metrohm) and a sample processor (AG 838, Metrohm). Anions were determined with a Metrosep A column (250 x 4.0 mm) and a mobile phase (buffer) with 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ at a flow rate of 0.7 mL/min. Cations were determined with a column (250 x 4.0 mm) (Metrosep C3, Metrohm) and nitric acid 3.5 mM as mobile phase. The injection volume of the sample was 20 µL and data collection was done by using the Processor software IC Net 2.3.

Reagents

Mobile phase for anions: Na_2CO_3 3.2 mM (339.2 mg Na_2CO_3 in 1000 mL of deionised water) and $NaHCO_3$ 1.0 mM (84 mg $NaHCO_3$ in 1000 mL of deionised water).

Mobile phase for cations: Nitric acid 3.5 mM (0.243 mL of nitric acid 65% in 1000 mL of deionised water).

Standard commercial solutions for anions and cations (Fluka).

Determination Procedure

Table 2.1 shows the calibration ranges for the different inorganic ions concentrations, therefore in some samples dilutions with distilled water were performed in order to fit to these ranges.

Table 2.1. Calibration ranges for the different inorganic ions (mg/L)

Anion	Low value	High value	Cation	Low value	High value
Cl^-	1.0	100	Li^+	0.05	5
NO_2^-	0.05	5	Na^+	1.5	150
NO_3^-	0.5	50	NH_4^+	0.1	10
Br^-	0.2	20	K^+	0.5	50
PO_4^{3-}	0.5	50	Mg^{2+}	0.5	50
SO_4^{2-}	1.5	150	Ca^{2+}	0.5	50
$S_2O_3^{2-}$	1.5	150			

2.1.5. Proteins

The proteins content is measured using a commercial kit for protein assays (TP0300, Sigma) based on the Lowry method and the modifications proposed by Peterson (Peterson, 1977).

The procedure is based on two chemical reactions. The first is the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein. This is followed by the reduction of the Folin and Ciocalteu's phenol reagent, which yields a purple color. Absorbance of the colored solution is read at a suitable wavelength between 500 nm and 800 nm. The protein concentration is determined from a calibration curve.

Reagents preparation

The Lowry Reagent Solution, The Folin and Ciocalteu's Phenol Reagent Working Solution, and the Protein Standard Solution are prepared following manufacturer's instructions.

Determination procedure

Prepare five Standard Tubes for calibration by diluting the Protein Standard Solution in water to a volume of 1.0 mL in appropriately labeled test tubes. Prepare the Sample test tubes and dilute to 1.0 mL with water. Label a test tube Blank and add 1.0 mL of water.

Add 1.0 mL of the Lowry Reagent Solution to Standard, Blank, and Sample tubes and mix well. Allow solutions to stand at room temperature for 20 minutes. With rapid and immediate

mixing, add 0.5 mL of the Folin and Ciocalteu's Phenol Reagent Working Solution to each tube. Allow color to develop for 30 minutes.

Transfer solutions to cuvets and measure the absorbance of the Standards and Sample tubes versus the Blank at a wavelength of 740 nm. Determine the protein concentration of the Sample tube from the calibration curve prepared from the plot of the absorbance values of the Standards versus their corresponding protein concentrations.

2.1.6. Other control parameters

pH

The pH is one of the key parameters measured in wastewater biological treatment systems, since its control is important to maintain the activity of the microorganisms involved in the different treatment processes. The pH measurements were performed with an electrode (52-03, Crison Instruments) equipped with an automatic compensatory temperature device (21-910-01, Crison Instruments) and connected to a measurement instrument (pH). The sensibility of the instrument is $\pm$ 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 pocket meter (Oxi 330i, WTW) with a membrane covered galvanic dissolved oxygen sensor (CellOx® 325, WTW) was used to measured the DO concentration in the laboratory scale reactors.

Conductivity and salinity

Conductivity, expressed in S/cm, was determined with a portable conductivity meter (model 524, Crison). The salinity was referred to the NaCl concentration of a hypothetical solution with the same conductivity as the studied sample; it is expresed in g/L of NaCl

2.2. BIOMASS CHARACTERISATION

2.2.1. Methods from the *Standard Methods*

Total Suspended Solids (TSS) Volatile Suspended Solids (VSS) and inorganic suspended solids (ISS) were determined according to the methods 2540 D (Total Suspended Solids Dried at 103-105 °C) and 2540 E (Fixed and Volatile Solids Ignited at 550 °C) reported in *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005).

2.2.2. Sludge Volume Index

The Sludge Volume Index (SVI) determination is defined in the Standard Methods for the Treatment of Water and Wastewater (APHA-AWWA-WPCF, 2005) as the volume in

millilitres occupied by 1 g of a suspension after 30 min settling. However, as suggested at the "*1st IWA-Workshop Aerobic Granular Sludge*" (Munich, 2004) and by Schwarzenbeck *et al.*, (2004) another parameter, the SVI_{10} (SVI after 10 minutes of settling) was used in all the chapters of this work instead of SVI_{30} (SVI after 30 minutes of settling) since it is more representative for granular biomass (de Kreuk *et al.*, 2007). A low SVI_{30} value does not necessarily imply sludge granulation and viceversa. Nevertheless a granular sludge bed does consolidate much faster, i.e., the terminal SVI_{30} is already reached after 10 minutes of settling.

2.2.3. Granules density

The biomass density (as mass of granules per volume of granules) was determined using the method described by Beun *et al.*, (2002) and modified in the laboratory of Environmental Engineering and Bioprocesses. First, a known amount of a homogeneous biomass sample is taken from the reactor and weight (W1) in a tare weight graduated cylinder (W2). Then, a known amount of liquid is removed from the sample (W3). 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 (W4). A known amount of the liquid above the settled granules is removed and a sample is taken from it. This fraction (Ab_1) and the original dextran blue solution (Ab_0) 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 water and not to biomass. Measuring also the dry weight of the reactor sample (VSS, APHA-AWWA-WPCF, 2005) the density of the granules can be calculated as g biomass per L of granules.

The density is calculated from equation 2.3:

$$\text{Density } (\text{gVSS/L}_{\text{granule}}) = \text{VSS} \times \frac{W_1 - W_2}{W_4 - W_2 - \left(\frac{Ab_0}{Ab_1} \times (W_4 - W_3) \right)} \qquad [2.3]$$

Being:

VSS: Volatile Suspended Solids concentration (g/L)

W_1: weight of the graduated cylinder with sample (g)

W_2: graduated cylinder weight (g)

W_3: weight of the graduated cylinder with sample after removal of liquid (g)

W_4: weight of the graduated cylinder after dextran blue addition (g)

Ab_0: Absorbance of the dextran blue solution (1 g/L)

Ab_1: Absorbance of the sample

2.2.4. Average diameter of the granules

Morphology of the granules was followed by image analysis. Images of the granular sludge were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (Stemi 2000-C, Zeiss). For digital image analysis the programme Image ProPlus® was used. A caption of the program is represented in Figure 2.1. The procedure of average diameter determination is as follows:

1. Definition of the range of colours corresponding to the area of interest in the image, i.e. the granules (Manual or Automatic),

2. Selection of the measurements of interest,

3. Exporting of the data of interest selected with the software (e.g., area, aspect, roundness, average diameter, etc.) to a worksheet (Excel)

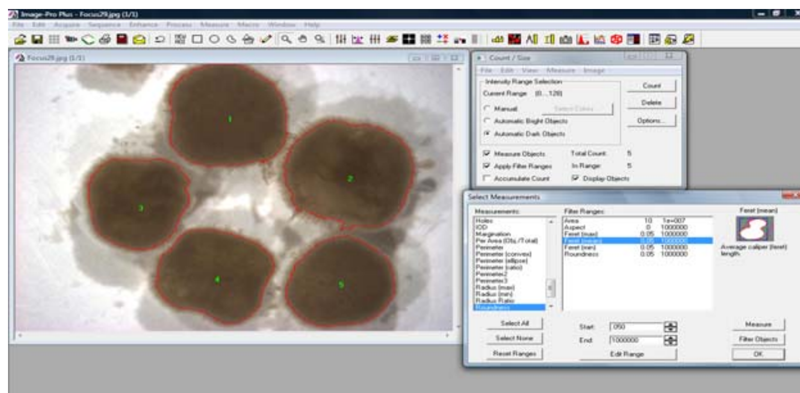


Figure 2.1. Original image of a sample of granules and area recognized by the software in red once the threshold levels are defined by the user.

The average diameter obtained from the programme corresponded to the mean feret diameter of the granules. The feret diameter is calculated as an average value from the shortest and the longest measured segments of each granule.

4. Utilization of the histogram tool (data analysis tool pack) to calculate the frequency and construction of the histogram. The average diameter can be calculated from a frequency, surface or volumetric distribution (Figure 2.2).

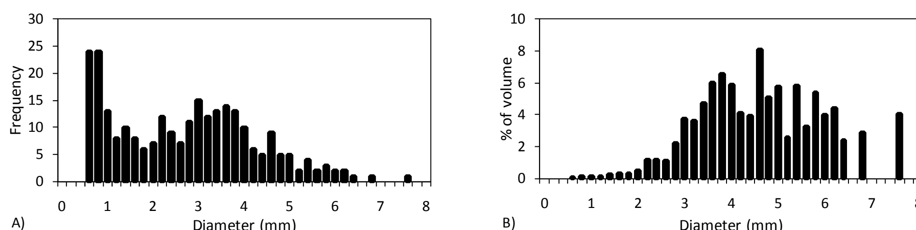


Figure 2.2. A) Frequency and B) volumetric distribution histograms of a granular sludge sample.

2.2.5. Electron Microscopy and Micro-analysis

Morphological studies of the biomass were performed with a scan electron microscope (Digital SEM 440, Leica) 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 a pH value of 7.4 and subsequently fixed with a solution of paraformaldehyde 4% in phosphate buffer for 3 hours. After fixation the sample was dehydrated using acetone solutions with increasing acetone concentrations (30, 50, 70 and 100%). Later the sample was shaded with gold and observed under the scan electron microscope.

To research the elemental composition of the granules a micro-analysis was carried out. The instrument used was the SEM LEO-435VP with a system of micro-analysis (EDX) at voltages varying in the range of 5 kV, 20 kV and 30 kV.

2.2.6. Respirometric assays

Activity tests were performed in order to measure the nitrifying capability of the biomass by means of a respirometric method (Lopez-Fiuza *et al.*, 2002; Mosquera-Corral *et al.*, 2005b) in a BOM5300 device. The maximum ammonia oxidizing was determined for aerobic granular sludge in Chapter 3. Respirometric batch experiments were performed at 20 °C and pH 7.0 in hermetically closed vials of 10 mL. Between 100 and 200 mg VSS/L were suspended in a buffer solution (1.25 mL/L traces; NaCl, 0.2 g/L; K_2HPO_4, 1.0 g/L; KH_2PO_4, 0.8 g/L; $MgSO_4$, 0.2 g/L; $MgCl_2 \cdot H_2O$, 0.3 g/L) of pH 7.0. The liquid and biomass mixture inside the vessel was bubbled with air for 15 min to reach the oxygen saturation (9.2 mg O_2/L at 20 °C). Meanwhile the software for the data acquisition (Labtech) was initiated and the two electrodes for oxygen measurement were calibrated to 100% oxygen saturation. To begin the experiment, aeration was removed and the oxygen probes were connected to the vessels and tightly closed avoiding the presence of bubbles.

The oxygen depletion was monitored during the time by means of the connection of the oxygen electrode to the data acquisition system (Figure 2.3). The endogenous respiration was measured during enough time to obtain the slope of the consumed oxygen (g O_2/L d). The

substrate (initial concentration of 60 mg NH_4Cl-N/L) was injected into the vial (10 µL) and then the slope of the oxygen consumption due to biomass activity was determined (g O_2/L d).

After the experiment, the solids content in each of the vials was determined according to the Standard Methods Ref. 2540 (APHA-AWWA-WPCF, 2005) (g VSS/L). Finally, the specific activity of the biomass is determined by dividing the oxygen consumption rate by the solids content, which can be referred to the specific substrate by the use of the stoichiometric coefficient.

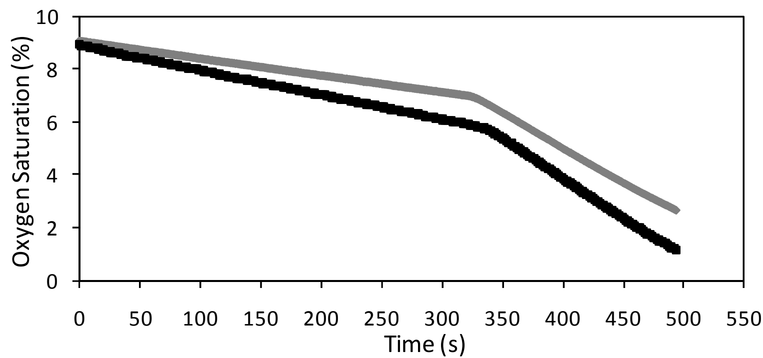


Figure 2.3. Oxygen depletion during endogenous respiration and substrate consumption.

2.3. CALCULATIONS

2.3.1. SBR operational cycle analysis

The activity of the different microbial populations present in the SBR was calculated using the concentration profiles during a whole cycle of operation, based in the procedure described by Mosquera-Corral *et al.* (2005) and according to the representation of Figure 2.4.

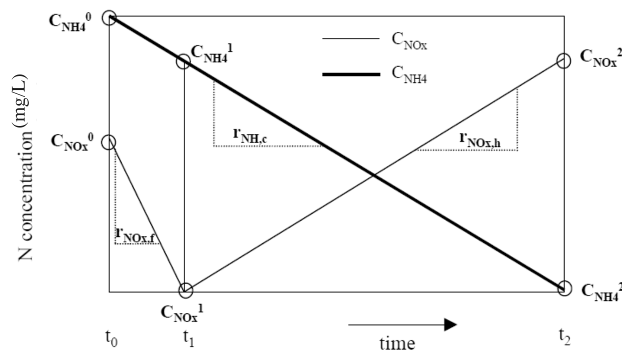


Figure 2.4. Schematic representation of the nitrogen compounds concentrations during the cycle and definition of the calculated parameters.

The consumption rates for ammonia (r_{NH4+}) and for nitrogen oxides (nitrite or nitrate) ($r_{NOx\ f}$) in the feast phase and the production rate of nitrogen oxides (nitrite or nitrate) ($r_{NOX\ h}$) during famine phase were calculated using the equations [2.4], [2.5] and [2.6].

$$r_{NH_4^+\ c}=\frac{C^0_{NH_4^+}-C^2_{NH_4^+}}{(t_2-t_0)/60} \qquad [2.4]$$

$$r_{NO_x^-\ f}=\frac{C^0_{NO_x^-}-C^1_{NO_x^-}}{(t_1-t_0)/60} \qquad [2.5]$$

$$r_{NO_x^-\ h}=\frac{C^1_{NO_x^-}-C^2_{NO_x^-}}{(t_1-t_2)/60} \qquad [2.6]$$

being t_0 the time at the beginning of the cycle, t_1 the time at the end of the feast phase and t_2 the time at the end of the famine phase and C_c^t the concentration of each compound (c) in a certain time (t), expressed in mg N/L. Specific rates were calculated by dividing the consumption rates by the solids concentration.

2.3.2. Estimation of the nitrogen assimilated and denitrified in a SBR

The main processes for nitrogen removal in the operated SBR were nitrogen assimilation for biomass growth and nitrification-denitrification for ammonia removal. The sum of the amount of nitrogen removed by both ways provides the value of N_R. In order to discern between the percentages of nitrogen removal achieved by each of these mechanisms a nitrogen balance was performed to the reactor to determine the amount of nitrogen used for growth. For each selected 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 2.7:

$$DW_p=\Delta X_r\cdot V_r+\overline{X}_{eff}\cdot Q\cdot\Delta_t \qquad [2.7]$$

Where:

DW_P: amount of produced biomass (g VSS)

ΔX_r: change of biomass concentration during each period (g VSS/L)

V_r: reactor volume (L)

$\overline{X}_{eff}$: average biomass concentration washed out in the effluent (g VSS/L),

Q: flow rate (L/d)

Δ_t: 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 ($DW_{\mathbf{N}}$) was calculated using equation 2.8 as:

$$DW_N = DW_P \cdot \frac{14 \text{ g-mol N}}{113 \text{ g-mol biomass}} \qquad [2.8]$$

The percentage of nitrogen assimilated ($N_{assimilated}$) is calculated by dividing the averaged amount of the nitrogen present in the produced biomass (DW_N) by the flow rate (Q) and the length of each period (Δt) and referring this obtained value to the nitrogen removed. Nitrogen removed by denitrification ($N_{denitrified}$) was calculated by the difference between $N_{removed}$ and $N_{assimilated}$.

2.3.3. Biomass production

The growth yield of microorganisms (Y_{obs}) in aerobic granules expressed in terms of gram of biomass produced per gram of organic matter removed (biomass production) was calculated for selected periods.

The amount of biomass produced was calculated according to equation 2.7 and the amount of organic matter removed was calculated from the experimental data obtained from the performance of the reactor in the selected period of time, as the difference between the average COD concentrations in the influent and effluent. Finally, the obtained amount of biomass is divided by the amount of COD removed according to equation 2.9:

$$Y_{obs} = \frac{DW_p}{(\overline{COD_{inf}} - \overline{COD_{eff}}) \cdot Q \cdot \Delta_t} \qquad [2.9]$$

Where:

Y_{obs}: growth yield of aerobic granules (g VSS /g COD)

DW_P: amount of produced biomass (g VSS)

$\overline{COD_{inf}}$ and $\overline{COD_{eff}}$: average COD concentration in the influent and effluent (g COD/L)

Q: flow rate (L/d)

Δ_t: length of each period (d)

2.3.4. Estimation of nitrogen removal rates in a CANON reactor

Ammonium and nitrite oxidation rates (AOR and NOR, respectively) and nitrogen removal rate by Anammox bacteria (NNR) in the reactor were estimated as kg N/(m^3 d) based on nitrogen balances and the stoichiometry of the Anammox process (Eqs. [2.10]-[2.13])

$$\Delta N = (NH_4^+\text{-}N_{inf}) - ((NH_4^+\text{-}N_{eff}) + (NO_2^-\text{-}N_{eff}) + (NO_3^-\text{-}N_{eff})) \qquad [2.10]$$

$$AOR=\frac{(NH_4^+\text{-}N_{inf})\text{-}(NH_4^+\text{-}N_{eff})\text{-}\frac{\Delta N}{2.04}}{HRT} \quad [2.11]$$

$$NOR=\frac{(NO_3^-\text{-}N_{eff})\text{-}\frac{0.26\ \Delta N}{2.04}}{HRT} \quad [2.12]$$

$$NRR=\frac{\Delta N}{HRT} \quad [2.13]$$

being HRT the hydraulic retention time (d), NH_4^+-N_{inf} the ammonium concentration in the influent (mg N/L) and NH_4^+-N_{eff}, NO_2^--N_{eff}, NO_3^--N_{eff} the ammonium, nitrite and nitrate concentrations in the effluent (mg N/L), respectively.

2.4. MICROBIOLOGICAL DETERMINATIONS

Molecular techniques based on the rRNA of *Bacteria* and *Prokaryotes* are presented in the next section. These techniques here described are not mutually exclusive and should be considered complementary in the analysis of microbial population, each of them with vantages and disadvantages. The FISH technique makes possible to identify microorganisms at any desired taxonomical level, depending on the specificity of the used probe. It is the only quantitative molecular biology technique, although quantification is either complex or tedious and subjective. The FISH technique was applied to cryosectioned granules in order to obtain the spatial distribution of bacteria in the granules and also to disrupted ones to check the presence of certain microorganisms. The DGGE is a rapid and simple method that provides characteristic band patterns for different samples, allowing quick sample profiling, while retaining the possibility of a more thorough genetic analysis by sequencing of particular bands. Cloning provides very precise taxonomical information, but is time consuming.

2.4.1. Identification of bacteria populations by FISH

The abundance of the different populations of microorganisms present in the sludge samples of the reactors was researched by Fluorescent *in situ* Hybridization (FISH). With this technique specific regions in 23S, 18S or 16S rRNA are detected with fluorescently labelled probes. If the corresponding domain, phylum, genus or species is present, the probe hybridizes to the targeted sequence and can be later detected microscopically.

This protocol was applied either to disrupted biomass or granules frozen and cut in slices. In each case, the objective and the preparation of the sample were different:

- In order to determine the presence of certain bacteria in the sample the granules were disrupted. In order to achieve the granular breakage, biomass was sonicated for 1 min at 65% of amplitude and frequency of 0.5 1/s using an ultrasonic device

(UP200s, Dr. Hielscher). The time of sonication was selected in order to achieve the breakage of the granules but not of the cells.

- Some granules were frozen and cut in slices in order to determine the stratification of bacteria along the granule. Entire granules were embedded in OCT reagent (Tissue-Tek; Miles Ind.) prior to their cryosectioning at -35 °C. Slides with a thickness ranging from 14 to 25 µm were cut at -20 °C, and these single sections were placed on the surface of poly-L-lysine coated microscopic slides.

Hybridization was performed according to the protocol described by Amann *et al.* (1990) with freshly prepared (not older than 24 h) 4% paraformaldehyde solution in phosphate buffer solution (PBS).

Reagents preparation:

PBS (3x): 0.49 g KH_2PO_4 are dissolved in 80 mL of milliQ water, 2.3 g of NaCl are added and pH is adjusted to 7.2. Finally, the volume is adjusted to 100 mL. PBS (1x) is prepared by a 1:3 dilution of PBS (3x) in milliQ water.

Fixative solution: First, 6.5 mL milliQ are heated to 60 °C and 0.4 g of paraformaldehyde are added. One drop of 1 M NaOH is added and the solution is shaken vigorously until it has nearly clarified (1-2 min). Then, 3.3 mL of PBS (3x) are added and the pH is adjusted to 7.2 with HCl (one drop 1 M HCl). Finally, the solution is filtered through 0.2 µm membrane filter.

Hybridization buffer: The buffer is prepared into a 2 mL eppendorf by mixing: 360 µL of NaCl 5 M and 40 µL of Tris/HCl (1 M) (pH 8.0). The percentage of formamide (% Formamide) of the hybridisation buffer is selected according the probe used (Table 2.2). Finally, 4 µL of sodiumdodecylsulfate 10% (w/v) are added to the mixture.

Table 2.2. Formamide and water added to the hybridisation buffer.

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

Washing buffer: The buffer is prepared into a 50 mL Falcon tube by mixing: 1 mL of Tris/HCl (pH 8.0) and 5 M NaCl and 0.5 M EDTA (pH 8.0) according to the percentage of formamide indicated (Table 2.3). The Falcon tube is filled up to 50 mL with milliQ water and the washing buffer is preheated at 48 °C prior to use.

Table 2.3. NaCl and EDTA added to the washing buffer.

% (v/v) Formamide	5 M NaCl (µL)	0.5 M EDTA (µL)
0	9000	-
5	6300	-
10	4500	-
15	3180	-
20	2250	500
25	1590	500
30	1120	500
35	800	500
40	560	500
45	400	500
50	280	500
55	200	500
60	80	500

Fixation of cells:

Biomass is washed in PBS (1x), then three volumes of fixative are added to one volume of suspension. The solution is kept on ice for 2 h and after that it is washed and the cells are resuspended in 1xPBS. Ethanol 98% (at -20 °C) is added to the biomass suspension in a ratio 1.25:1. Samples are stored at -20 °C.

Immobilization of cell on microscope slides:

Fixed biomass suspension is spread in each well of a coated Teflon/glass microscope slide (10 µL). The slide is dried at 46 °C for 10 min. Cells are dehydrated by successive passage through 50%, 80% and 98% ethanol (3 min each) and dried at under air.

Hybridization:

The hybridization buffer is prepared and kept at room temperature. The hybridization tube is prepared by putting a folding tissue into a 50 mL Falcon tube.

Part of the hybridisation buffer (10 µL) is pipetted into the wells of the slides with the biomass and the rest is poured onto the tissue of the Falcon tube and the FISH probe is added (1 µL of stock solution with a final concentration 30 ng/µL for CY3 and CY5-labeled probes and 50 ng/µL for FITC labelled probes). The slide is transferred into the hybridization tube and incubated for 1.5 h at 46 °C. In the meantime, the washing buffer is prepared and preheated in a waterbath at 48 °C.

Washing:

This step should be performed rapidly: The slide is transferred into washing buffer and incubated 15 min at 48 °C. Then the slide is removed from the washing buffer and dipped into cold milliQ water for few seconds and dried under air.

Microscopy and image acquisition:

Slide wells are embedded with Vectashield H-1200 (amplifies fluorescence, avoids fading and contains DAPI) and the cover slip is put on the slide.

Specimens can now be analysed with fluorescence microscope

- For analysis of the slides with disrupted samples an epifluorescence microscope (Axioskop 2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics) was used. Acquisition software was RSI image v 1.7.3. (Roper Scientific, Inc.)
- For analysis of the slides with sliced granules a TCS-SP2 confocal laser scanning microscope (Leica, Germany), equipped with HeNe laser for the detection of the fluorochromes Cy3 and Cy5 and Ar laser for the detection of the fluorochrome FITC was used. Acquisition software was Leica Confocal Software Lite®.

Paired images (FITC, Cy3, CY5 or DAPI) of each field of view are stored in TIFF format. The image software Serif Photo plus was used to merge two or three of the images depending of the requirements (Figure 2.5). The blend mode "ligthen" was chosen.

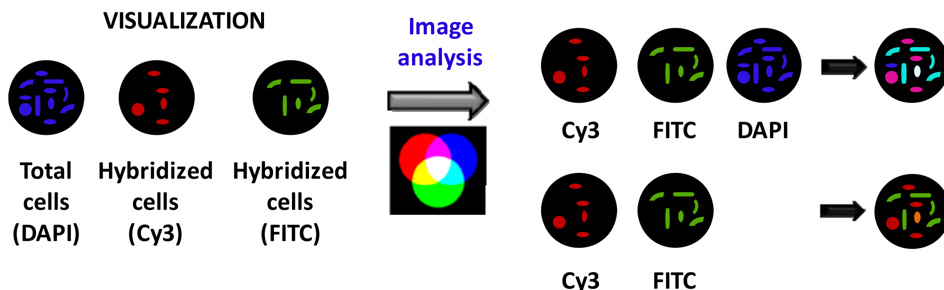


Figure 2.5. Schematic representation of the colours resulting after merging the different images.

Quantification:

Quantification of the bacterial population was based on the use of *daime* (digital image analysis in microbial ecology) software by measuring the relative abundances (fractions of the total biovolume) of probe-labelled populations in digital images (Daims *et al.*, 2006). The quantification was performed by comparison of the positive area obtained with a specific probe with the area corresponding to the control: DAPI or EUBmix (a mixture of EUB338, EUB338 II and

EUB338 III). Digital images from 20 to 30 different fields of view were obtained at randomly chosen positions.

This program does not quantify absolute cell numbers, but determines the *biovolume fraction* of the specifically labelled target population relative to the biovolume of the total biomass (Figure 2.6). Although one of the recommendations of *daime* software is its use with images acquired by using a confocal microscope, in this thesis, *daime* software was used with images acquired by using an epifluorescence microscope, with the objective of having an approximate idea of the percentages of certain populations.

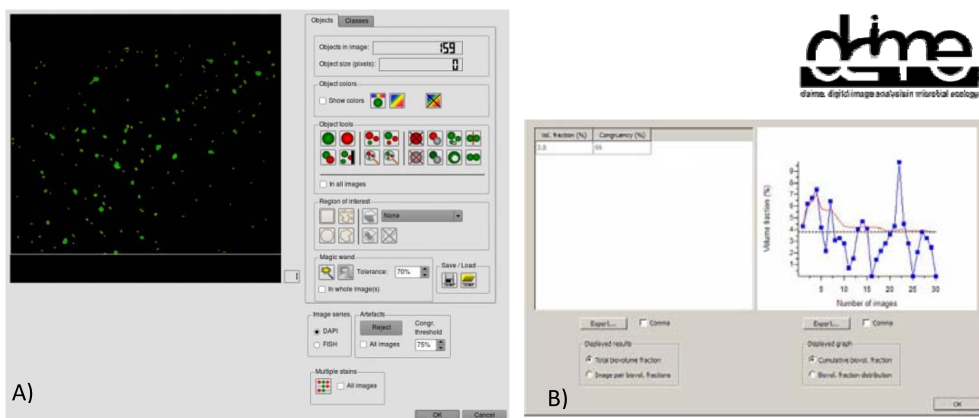


Figure 2.6. Screenshots of the daime software. A) The object editor and B) an example of results of a biovolume fraction measurement

FISH probes

A schematic tree reflecting the most important probes applied in this study, indicating the main bacteria detected by each probe, is shown in Figure 2.7. The probes applied in this study are listed and detailed in Table 2.4.

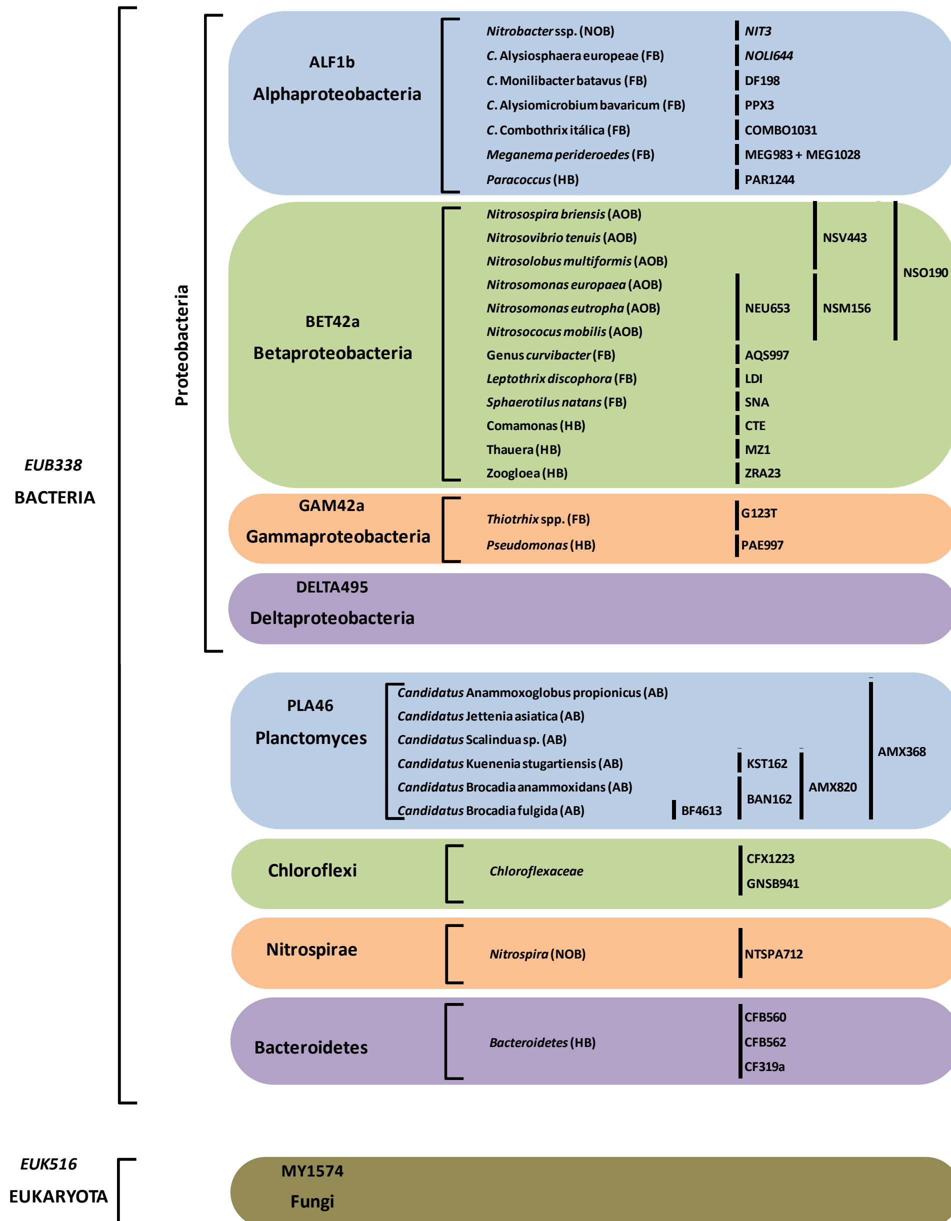


Figure 2.7. Different probes for Bacteria and Eukaryota superkingdom applied in this thesis (AOB: ammonium-oxidizing bacteria, NOB: nitrite-oxidizing bacteria, HB: heterotrophic bacteria, FB: filamentous bacteria and AB: Anammox bacteria).

Table 2.4. Probes used for fluorescent *in situ* hybridisation and the formamide (F) concentration used during hybridization.

Probe	Target site	Probe sequence (5'→3')	% F	Target organisms	Ref[e]
EUB338I[d]	338-355[a]	GCTGCCTCCCGTAGGAGT	0-50	Bacteria domain	[1]
EUB338II	338-355[a]	GCAGCCACCCGTAGGTGT	0-50	*Planctomycetales*	[2]
EUB338III	338-355[a]	GCTGCCACCCGTAGGTGT	0-50	*Verrucomicrobiales*	[2]
ALF1B	19-35[a]	CGTTCGYTCTGAGCCAG	20	*Alphaproteobacteria*	[3]
NIT3	1035-1052[a]	CCTGTGCTCCATGCTCCG	40	*Nitrobacter* spp.	[4]
Comp.		CCTGTGCTCCAGGCTCCG			
NOLI644	644-660[a]	TCCGGTCTCCAGCCACA	35	*Candidatus* Alysiosphaera europea	[5]
PPX3	1428-1445[a]	TGGCCCACCGGCTTCGGG	50	*Candidatus* Alysiomicrobium bavaricum	[5]
DF198	198-217[a]	ATCCCAGGGCAACATAGTCT	35	*Candidatus* Monilibacter batavus	[6]
COMBO1031	1031-1050[a]	CACCTGCAGTGGCCTCCCGA	35	*Candidatus* Combothrix itálica	[7]
MEG983	983-1001[a]	CGGGATGTCAAAAGGTGG	35	*Meganema perideroedes*	[8]
MEG1028	1028-1046[a]	CTGTCACCGAGTCCCTTGC	35	*Meganema perideroedes*	[8]
PAR1244	1244-1262[a]	GGATAACCCACTGTCACC	20	*Paracoccus*	[9]
BET42a	1027-1043[b]	GCCTTCCCACTTCGTTT	35	*Betaproteobacteria*	[3]
Competitor		GCCTTCCCACATCGTTT			
Nso190	189-207[a]	CGATCCCCTGCTTTTCTCC	55	Betaproteobacterial ammonia-oxidizing bacteria	[10]
NSV443	444-462[a]	CCGTGACCGTTTCGTTCCG	30	*Nitrosospira* spp.	[10]
Nsm156	156-174[a]	TATTAGCACATCTTTCGAT	5	*Nitrosomonas* spp., *Nitrosococcus* mobilis	[10]
NEU653	653-670[a]	CCCCTCTGCTGCACTCTA	40	Most halophilic and halotolerant *Nitrosomonas* spp.	[11]
Competitor		TTCCATCCCCCTCTGCCG			
Curvi997	997-1014[a]	CTCTGGTAACTTCCGTAC	35	*Curvibacter delicatum*; *Pseudomonas lanceolata* and few more Betaproteobacteria	[12]
Competitor1		CTCTGGCAACTTCCGTAC			
Competitor2		CTCTGGTCACTTCCGTAC			
LDI	649-666[a]	CTCTGCCGCACTCCAGCT	35	*Leptothrix discophora*	[13]
CTE	659-676[a]	TTCCATCCCCCTCTGCCG	20	*Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp., *Curvibacter* spp.	[14]
MZ1	646-664[a]	TCTGCCGTACTCTAGCCTT	45	*Thauera* spp.	[15]
ZRA23	647-664[a]	CTGCCGTACTCTAGTTAT	35	Most members of the *Zoogloea* lineage, not *Z. resiniphila*	[16]
SNA	656-673[a]	CATCCCCCTCTACCGTAC	45	*Sphaerotilus natans*	[17]
GAM42a	1027-1043[b]	GCCTTCCCACATCGTTT	35	*Gammaproteobacteria*	[3]
Competitor		GCCTTCCCACTTCGTTT			
G123T	697-714[a]	CCTTCCGATCTCTATGCA	40	*Thiothrix eikelboomii, T. nivea, T. unzii, T. fructosivorans, T. Defluvii*	[18]
Competitor		CCTTCCGATCTCTACGCA			
PAE997	997-1014[a]	TCTGGAAAGTTCTCAGCA	0	*Pseudomonas* spp.	[19]

CFX1223	1223-1242[a]	CCATTGTAGCGTGTGTGTMG	35	phylum *Chloroflexi*	[20]
GNSB941	941-957[a]	AAACCACACGCTCCGCT	35	phylum *Chloroflexi*	[21]
CF319a	319-336	TGG TCC GTG TCT CAG TAC	35	most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*	[22]
CFB560	560-575	WCCCTTTAAACCCART	30	subgroup of *Bacteroidetes*	[23]
CFB562	562-580	TACGYWCCCTTTAAACCCA	30	subgroup of *Bacteroidetes*	[23]
Ntspa712	712-732[a]	CGCCTTCGCCACCGGCCTTCC	35	Most members of phylum Nitrospirae	[24]
Competitor		CGCCTTCGCCACCGGGTTCC			
PLA46	46-63[a]	GACTTGCATGCCTAATCC	20	Planctomycetes	[25]
Amx368	368-385[a]	CCTTTCGGGCATTGCGAA	15	all anammox bacteria	[26]
Amx820	820-841[a]	AAAACCCCTCTACTTAGTGCCC	35	*Candidatus* Brocadia anammoxidans	[27]
Kst157	157-174[a]	GTTCCGATTGCTCGAAAC	25	*Candidatus* Kuenenia stuttgartiensis	[27]
Ban162	162-179[a]	CGGTAGCCCCAATTGCTT	40	*Candidatus* Brocadia anammoxidans	[27]
Bfu613	613-636[a]	GGATGCCGTTCTTCCGTTAAGCGG	30	*Candidatus* "Brocadia fulgida"	[28]
EUK516	502-517[c]	ACCAGACTTGCCCTCC	25	*Eukarya*	[1]
MY1574	1474-1489[c]	TCCTCGTTGAAGAGC	20	*Eumycota (*Fungi*)*	[29]

[a] 16s RNA position, [b] 23s RNA position, [c] 18s RNA position

[d] EUB338mix probe is an equimolar mixture of probes EUB338I, EUB338II and EUB338III

[e] [1] Amann *et al.*, 1990; [2] Daims *et al.*, 1999; [3] Manz *et al.*, 1992; [4] Wagner *et al.*, 1996; [5] Snaidr *et al.*, 2002; [6] Nittami *et al.*, 2009; [7] Levantesi *et al.*, 2004; [8] Thomsen *et al.*, 2006; [9] Neef *et al.*, 1996, [10] Mobarry *et al.*, 1996;[11] Wagner *et al.*, 1995; [12] Thomsen *et al.*, 2004; [13] Wagner *et al.*, 1994; [14] Schleifer *et al.*, 1992; [15] Lajoie *et al.*, 2000; [16] Rosselló-Mora *et al.*, 1995; [17] Wagner *et al.*, 1994; [18] Kanagawa *et al.*, 2000; [19] Amann *et al.*, 1996 ; [20] Bjornsson *et al.*, 2002; [21] Gich *et al.*, 2001; [22] Manz *et al.*, 1996; [23] O'Sullivan *et al.*, 2002; [24] Daims *et al.*, 2001; [25] Neef *et al.*, 1998; [26] Schmid *et al.*, 2003; [27] Schmid *et al.*, 2001; [28] Kartal *et al.*, 2008; [29] Baschien, 2003.

2.4.2. DNA extraction

Biomass was harvested directly from the SBR and sonicated for 1 min at 65% of amplitude using an ultrasonic device (UP200s, Dr. Hielscher) to achieve the granular biomass breakage. DNA was extracted using the MoBio Power Soil™ kit (MoBio Laboratories) according to the manufacturer's protocol. DNA was then suspended in 50 µL ultrapure water, and kept at 4 °C until further analysis.

2.4.3. PCR amplification

PCR mixes used in the reactions were prepared in a laminar flow cabinet. All the material was sterilized.

The extracted DNA was PCR amplified using an automated thermal cycler (Applied Biosystems). All the primers used in this work were synthesized and purified by Thermo-

Hybaid (Germany). The used concentration was 10 µM. The primers used and the PCR conditions are shown in Table 2.5.

In Chapter 4, the reactions were performed in a volume of 50 µL, using 1.25 U of Taq (TaKaRa ExTaq™ Hot Start; TaKaRa Bio Inc., Japan), 1x ExTaq Buffer (2 mM $MgCl_2$), 200 µM of deoxynucleoside triphosphate, 0.5 µM of primers and 1 µL of template DNA.

In Chapter 7, the reactions were conducted in a final volume of 25 µL, using GoTaq Green Master Mix (Promega) and 1 µL of template DNA.

The PCR products were checked by electrophoresis by loading 4 µL of the reaction product in 1% agarose gel and fragment sizes were estimated using a GeneRuler™ DNA Ladder Mix (Fermentas).

Table 2.5. PCR primers and PCR conditions used.

Primer	Sequence (5'→3')	Target	PCR conditions	Ref.
PLA46F	GACTTGCATGCCTAATCC	16s RNA gene of Planctomycetes	5 min at 96 °C, followed by 35 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.	[1]
630R	CAKAAAGGAGGTGATCC			
amoA 1F	GGGGTTTCTACTGGTGGT	amoA gene of AOB	5 min at 96 °C, followed by 35 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.	[2]
amoA 2R	TCCCCTCTGCAAAGCCTTCTTC			
CTO189F*	GGAGRAAAGYAGGGGATCG	16s RNA gene of Betaproteobacteria AOB	5 min at 96 °C, followed by 25 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.	[3]
CTO654R	CTAGCYTTGTAGTTTCAAACGC			
341F*	CCTACGGGAGGCAGCAG	16s RNA gene of Eubacteria	9 min at 95 °C, followed by 25 cycles of 60 s at 94 °C, 60 s at 55 °C, 90 s at 72 °C and 10 min of final extension at 72 °C.	[4]
907R	CCGTCAATTCCTTTGAGTTT			
616F	AGAGTTTGATYMTGGCTCAG	16s RNA gene of Eubacteria	5 min at 96 °C, followed by 35 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.	[5]
630R	CAKAAAGGAGGTGATCC			

*GC Clamp added for DGGE-PCR: 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG- 3'.

[1] Schmid *et al.*, 2003; [2] Nicolaisen and Ramsing, 2002; [3] Kowalchuk *et al.*, 1997; [4] Yu and Morrison, 2004; [5] Juretschko *et al.*, 1998.

After the amplification of the extracted DNA and prior to the DGGE the following protocol was used to precipitate the sequencing reaction products:

1. For 50 µL of PCR product, add 4.5 µL CH3COONa 3 M and 90 µL of cold 100% ethanol (- 20 °C).
2. Mix and incubate at room temperature for 60 minutes.

3. Centrifuge at 12000 rpm for 30 minutes and discard very carefully the supernatant by using a micropipette.
4. Incubate the pellet at 37 °C until it dries.
5. The dry pellet can be either stored at -20°C or resuspended in 15 μL of water.

2.4.4. Agarose gel electrophoresis

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

2.4.5. Denaturing gradient gel electrophoresis

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

The amplified sequences were separated by DGGE using an Ingeny phorU system (Ingeny).

In order to determine the appropriate denaturing range a previous DGGE was prepared at 20-80% denaturing gradient. The polyacrylamide gel (6% [wt/vol]) contained the linear formamide/urea gradient overlaid with a non-denaturing stacking gel. Once the samples were located in the gel, narrower gradients were selected to sharpen detection of different DNA sequences.

Reagents preparation

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

Stock solutions: aliquots with different percentages of urea/formamide were prepared and kept refrigerated. For each run of the gel is necessary to take 22 mL of the low concentrate solution (i.e. 20%) and the high concentrated solution (i.e. 80%). Reagent volumes needed are presented in Table 2.6.

Table 2.6. DGGE stock concentration and reagent volumes for 6% polyacrilamide gels

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

Making a DGGE gel

1. Clean the glasses, assemble and clamp the front and back glass plates with the spacers in between and then place the comb according to the Ingeny instructions. Wash out the gradient former.
2. Prepare the low and high concentrated solutions, and add 15 µL of TEMED (N,N,N',N'-tetramethylenediamine) and 100 µL of 10 % APS (Ammonium Persulfate).
3. Mix in the tube by inverting it a couple of times and pour its content into the open side of the gradient former. With the help of a gradient former and a peristaltic pump, the two solutions are introduced into the gel sandwich.
4. Allow the gel to polymerize for about 1–2 hours at room temperature.
5. Add 10 µL of TEMED and 40 µL of APS to 8 mL of 0% DGGE solution. Mix by inverting several times and pour into the gradient former.

Sample loading, gel running and visualization

1. Put the polymerized gel sandwich inside the electrophoresis tank preheated to 60 °C with TAE 1x buffer, and check if the upper chamber is filled up.
2. Prepare the samples with 1/4 (vol/vol) of loading buffer.
3. Run the gel at 100 V for about 15 hours at 60 °C
4. Gels were stained with SYBR-Green solution (Invitrogen) in TAE 1x buffer for 30 minutes and visualized under UV transillumination in the LIAS *Xlite* system (Avegene).

After visualization of the gel, specific gel bands were excised with a sterilized scalpel, dissolved in 30 µL Milli-Q water and stored at 4 °C overnight to further reamplification by PCR using the same primers of first PCR without the GC clamp.

Digitized DGGE images were analyzed using the program Gel 2K (Svein Norland, Dept. of Biology, University of Bergen), which determines the presence or absence of bands (band patterns) based on grey level units and pixel intensity. The DGGE patterns were tested for similarity by using the Jaccard-Index that calculates the resemblance matrix. This matrix was used for the creation of a cladogram, which classifies together the closest obtained profiles.

2.4.6. Cloning and sequencing

The amplification of any gene from environmental samples generates a mixture of fragments from all the members of the community holding this gene. To identify the different genes, they must be previously isolated. Cloning with *Escherichia coli* was used to separate a mixture of 16S or *amoA* gene fragments to further sequence and compare with those from the databases. Cloning is based on inserting the mixture of amplicons in a vector. Every vector containing one single gene or gene fragment (insert) is then transformed into an *E. coli* strain. Spread plating in Petri dishes yields colonies that carry one single insert. A certain modification in the genotype of the cells makes it possible their use for the blue/white screening of colonies on bacterial plates containing X-Gal. By selecting the colonies and growing them independently every insert is obtained individually.

PCR products were ligated and transformed into *Escherichia coli* using the pGEM-T Easy vector system kit (Promega) according to the manufacturer's instructions. Cells containing a plasmid with an insert form white colonies. The pGEM®–T Easy Vector (Promega) also confers resistance to ampicillin. The plating on growth medium containing this antibiotic will only permit the growth of cells containing the vector (blue and white colonies). Of those, only the white ones also contain the insert. White colonies were recovered from the Petri dish with a toothpick and let them grow overnight at 37 °C in LB medium with ampicillin (Figure 2.8).

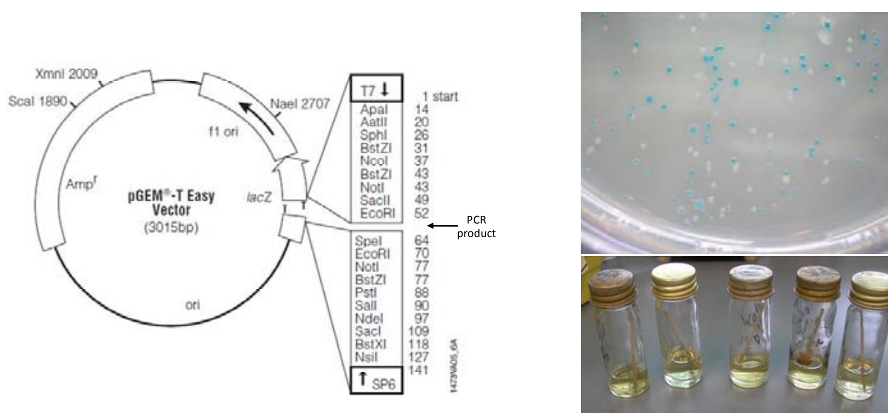


Figure 2.8. A) pGEM®–T Easy Vector scheme showing the insert location and several targets for enzymatic restriction. B) White/blue screening of colonies and C) transformant cells in flasks before the growth.

Plasmid DNA was isolated with the GeneJET Plasmid Miniprep kit (Fermentas) according to the manufacturer's protocol and digested with 5U EcoRI (Fermentas) enzyme in EcoRI buffer for 2 h at 37 °C. Restriction enzymes recognize a specific sequence in the DNA chain as a target, and cut it in two particular sites (positions 52 and 70 pf pGEM-T Easy vector). The digestion products were examined for an insert with the expected size by electrophoresis in 1% agarose gel.

The insert was directly amplified from the transformant colonies by using 2 μL of cell suspension as a template in a PCR reaction. The sequences of the 16S rRNA gene fragments were determined by using M13 forward (M13F 5′-GTAAAACGACGGCCAG-3´) and reverse (M13R 5′-CAGGAAACAGCTATGAC-3´) primers targeting vector sequences adjacent to the multiple cloning site. The sequences of the AmoA gene were determined by using M13 forward primer (Suzuki *et al.*, 2001).

Sequencing was done with BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems). The reaction mixtures were analyzed with the 3700 DNA analyzer (Applied Biosystems). In certain cases, primers T7 (5′-TAATACGACTCACTATAGGG-3´) and SP6 (5′-ATTTAGGTGACACTATAG-3´) were also used in sequencing.

2.4.7. Sequence analysis

Sequences were manually inspected and refined by using the software BioEdit Sequence Alignment Editor v7.0.5 (Ibis Therapeutics).

Obtained sequences were compared to reference sequences obtained from the databases EMBL-Bank (EMBL, European Molecular Biology Laboratory) and GenBank (NCBI, National Center for Biotechnology Information). Sequence similarities were checked using the BLAST (Basic Local Alignment Search Tool) software (Altschul *et al.*, 1990), which is available at the web pages:

http://us.expasy.org/tools/blast/

http://www.ncbi.nlm.nih.gov/BLAST/

In Chapter 4, sequences were aligned with reference sequences obtained from GenBank using MAFFT (Katoh and Toh, 2008). The alignment obtained was edited using MacClade software (Maddison and Maddison, 2003) and transferred to jMODELTEST software version 3.6 (Posada and Crandall, 1998) as a guide to determine the best-fit maximum likelihood model for the edited alignment using the Akaike information criterion. Rate variation across sites, assuming a gamma distribution of 0.4020 and a proportion of invariable sites of 0.2270 were allowed. The best-fit models of nucleotide evolution for A, C, G and T were 0.2404, 0.2297, 0.3224 and 0.2076, respectively, with the rate matrix of the substitution model being 0.9002 (A/C), 1.8993 (A/G), 1.0584 (A/T), 0.7012 (C/G), 3.3843 (C/T) and 1.0000 (G/T). These

calculated parameters were incorporated into software PHYML (Guidon and Gascuel, 2003), which uses a single, fast, and accurate algorithm to estimate large phylogenies by ML. Finally, the trees created by PHYML were edited using the FIGTREE v1.1.2 software (http://tree.bio.ed.ac.uk/software/figtree/).

In Chapter 7, forward and reverse retrieved sequences of the 16S rRNA gene were manually inspected using the Contig Express software from the Vector NTI Suite 6.0 (Informax). Overlapping fragments of the 16S rRNA gene of *Planctomycetes* were assembled into a single contiguous sequence. Multiple sequence alignment of the sequences was carried out with ClustalW by using the tool implemented in MEGA4 software (Tamura *et al.*, 2007). Phylogenetic trees were constructed in MEGA4 by using the Neighbor-Joining method and *bootstrap* test with 100 replications. The Neighbour Joining method (Saitou and Nei, 1987) is a method for re-constructing phylogenetic trees, and computing the lengths of the branches of this tree. In each stage, the two nearest nodes of the tree are chosen and defined as neighbours in the tree (Figure 2.9). This is done recursively until all of the nodes are paired together. The bootstrap test is a way of testing the reliability of the dataset (Felsenstein, 1985) and is based on the creation of pseudoreplicate datasets by randomly resampling the original matrix. This method creates new matrices of the same size as the original, and the frequency with which a given branch is found, is recorded as the bootstrap proportion. These proportions can be used as a measure of the reliability of individual branches in the original tree. Finally, the consensus tree, which incorporates the branches with higher bootstrap values, is built. The bootstrap value indicates how many times, regarding the total number of replicates, a branch appears at a given position.

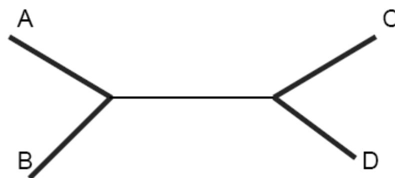


Figure 2.9. Nodes A and B and nodes C and D are neighbours (connected by only one internal node).

The presented methodology and calculations, together with those reported in the Standard Methods, will be used during the rest of this manuscript to obtain the data from the different research works. In each chapter the most important methods will be mentioned.

2.5. REFERENCES

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* 215(3), 403-410.

Amann R., Binder B.J., Olson R.J., Chisholm S.W., Devereux R. and Stahl D.A. (1990). Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial-populations. *Applied and Environmental Microbiology,* 56(6), 1919-1925.

Amann R., Ludwig W., Schulze R., Spring S., Moore E. and Schleifer K.H. (1996). rRNA-targeted oligonucleotide probes for the identification of genuine and former pseudomonads. *Systematic and Applied Microbiology,* 19(4), 501-509.

APHA-AWWA-WPCF (2005) Standard methods for the examination of water and wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Baschien C. (2003) Development and evaluation of rRNA targeted in situ probes and phylogenetic relationships of freshwater fungi, Technical University of Berlin, Berlin.

Beun J.J., van Loosdrecht M.C.M. and Heijnen J.J. (2002). Aerobic granulation in a sequencing batch airlift reactor. *Water Research,* 36(3), 702-712.

Bjornsson L., Hugenholtz P., Tyson G.W. and Blackall L.L. (2002). Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal 9. *Microbiology,* 148(8), 2309-2318.

Daims H., Bruhl 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(3), 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(11), 5273-5284.

Daims H., Lucker S. and Wagner M. (2006). daime, a novel image analysis program for microbial ecology and biofilm research. *Environmental Microbiology,* 8(2), 200-213.

de Kreuk M., Kishida N. and van Loosdrecht M.C.M. (2007). Aerobic granular sludge: state of the art. *Water Science and Technology,* 55 (8-9), 75-81.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39(4), 783-791.

Gich F., Garcia-Gil J. and Overmann J. (2001). Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. *Archives of Microbiology,* 177(1), 1-10.

Guidon S. and Gascuel O. (2003). PHYML-A single, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood *Systematic Biology,* 52(5), 696-704.

Juretschko S., Timmermann G., Schmid M., Schleifer K.H., Pommerening-Roser A., Koops H.P. and Wagner M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. *Applied and Environmental Microbiology,* 64(8), 3042-3051.

Kanagawa T., Kamagata Y., Aruga S., Kohno T., Horn M. and Wagner M. (2000). Phylogenetic analysis of and oligonucleotide probe development for Eikelboom Type 021N filamentous bacteria isolated from bulking activated sludge. *Applied and Environmental Microbiology,* 66(11), 5043-5052.

Kartal B., van Niftrik L., Rattray J., van de Vossenberg J.L.C.M., Schmid M.C., Sinninghe Damste J., Jetten M.S.M. and Strous M. (2008). Candidatus "Brocadia fulgida": an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiology Ecology,* 63(1), 46-55.

Katoh K. and Toh H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics,* 9(4), 286-298.

Kowalchuk G.A., Stephen J.R., DeBoer W., Prosser J.I., Embley T.M. and Woldendorp J.W. (1997). Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Applied and Environmental Microbiology,* 63(4), 1489-1497.

Lajoie C.A., Layton A.C., Gregory I.R., Sayler G.S., Taylor D.E. and Meyers A.J. (2000). Zoogleal clusters and sludge dewatering potential in an industrial activated-sludge wastewater treatment plant. *Water Environment Research,* 72, 56-64.

Levantesi C., Beimfohr C., Geurkink B., Rossetti S., Thelen K., Krooneman J., Snaidr J., van der Waarde J. and Tandoi V. (2004). Filamentous Alphaproteobacteria associated with bulking in industrial wastewater treatment plants. *Systematic and Applied Microbiology,* 27(6), 716-727.

Lopez-Fiuza J., Buys B., Mosquera-Corral A., Omil F. and Mendez R. (2002). Toxic effects exerted on methanogenic, nitrifying and denitrifying bacteria by chemicals used in a milk analysis laboratory. *Enzyme and Microbial Technology,* 31(7), 976-985.

Maddison D.R. and Maddison W.P. (2003) MacClade 4: Analysis of phylogeny and character evolution.Version 4.06, Sinauer Associates, Sunderland, Massachusetts.

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(4), 593-600.

Manz W., Amann R., Ludwig W., Vancanneyt M. and Schleifer K.-H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology,* 142(5), 1097-1106.

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology,* 62(6), 2156-2162.

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

Mosquera-Corral A., Gonzalez F., Campos J.L. and Mendez R. (2005b). Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds. *Process Biochemistry,* 40(9), 3109-3118.

Muyzer G., Dewaal E.C. and Uitterlinden A.G. (1993). Profiling of Complex Microbial-Populations by Denaturing Gradient Gel-Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes-Coding for 16S Ribosomal-Rna. *Applied and Environmental Microbiology,* 59(3), 695-700.

Neef A., Zaglauer A., Meier H., Amann R., Lemmer H. and Schleifer K.H. (1996). Population analysis in a denitrifying sand filter: conventional and in situ identification of Paracoccus spp. in methanol-fed biofilms. *Applied and Environmental Microbiology,* 62(12), 4329-4339.

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-Uk,* 144, 3257-3266.

Nicolaisen M.H. and Ramsing N.B. (2002). Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. *Journal of Microbiological Methods,* 50(2), 189-203.

Nittami T., McIlroy S., Seviour E.M., Schroeder S. and Seviour R.J. (2009). Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III Defluviicoccus. *Systematic and Applied Microbiology,* 32(7), 480-489.

O'Sullivan L.A., Weightman A.J. and Fry J.C. (2002). New Degenerate Cytophaga-Flexibacter-Bacteroides-Specific 16S Ribosomal DNA-Targeted Oligonucleotide Probes Reveal High Bacterial Diversity in River Taff Epilithon. *Applied and Environmental Microbiology,* 68(1), 201-210.

Peterson G.L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical Biochemistry,* 83(2), 346-356.

Posada D. and Crandall K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics,* 14(9), 817-818.

Rosselló-Mora R.A., Wagner M., Amann R. and Schleifer K.H. (1995). The abundance of *Zoogloea ramigera* in sewage treatment plants. *Applied and Environmental Microbiology,* 61(2), 702-707.

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

Schleifer K.H., Amann R., Ludwig W., Rothermund C., Springer N., Dorn S., Galli E., Silver S. and Witholt B. (1992). Nucleic acid probes for the identification and in situ detection of pseudomands in *Pseudomans: Molecular biology and biotechnology*. pp. 127-135, American Society for microbiology.

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(7), 450-459.

Schmid M., Walsh K., Webb R., Rijpstra W.I.C., van de Pas-Schoonen K., Verbruggen M.J., Hill T., Moffett B., Fuerst J., Schouten S., Damste J.S.S., Harris J., Shaw P., Jetten M. and Strous M. (2003). Candidatus "Scalindua brodae", sp nov., Candidatus "Scalindua wagneri", sp nov., two new species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology,* 26(4), 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.

Snaidr J., Beimfohr C., Levantesi C., Rossetti S., van der Waarde J., Geurkink B., Eikelboom D., Lemaitre M. and Tandoi V. (2002). Phylogenetic analysis and in situ identification of "Nostocoida limicola"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. *Water Science and Technology,* 46(1-2), 99-104.

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

Stahl D.A. and Amann R. (1991). Development and application of nucleic acid probes in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (eds), pp. 205-248, Jon Wiley & Sons Ltd., Chichester.

Suzuki Y., Yoda T., Ruhul A. and Sugiura W. (2001). Molecular cloning and characterization of the gene coding for Azoreductase from Bacillus sp. OY1-2 isolated from soil. *Journal of Biological Chemistry,* 276(12), 9059-9065.

Tamura K., Dudley J., Nei M. and Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution,* 24(8), 1596-1599.

Thomsen T.R., Nielsen J.L., Ramsing N.B. and Nielsen P.H. (2004). Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. *Environmental Microbiology,* 6(5), 470-479.

Thomsen T.R., Blackall L.L., de Muro M.A., Nielsen J.L. and Nielsen P.H. (2006). Meganema perideroedes gen. nov., sp. nov., a filamentous alphaproteobacterium from activated sludge. *International Journal of Systematic and Evolutionary Microbiology,* 56(8), 1865-1868.

Wagner M., Amann R., Kampfer P., Assmus B., Hartmann A., Hutzler P., Springer N. and Schleifer K.H. (1994). Identification and in-situ detection of Gram-negative filamentous bacteria in activated-sludge. *Systematic and Applied Microbiology,* 17(3), 405-417.

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(2), 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(1-2), 237-244.

Weatherburn M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry,* 39(8), 971-974.

Yu Z. and Morrison M. (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-Denaturing Gradient Gel Electrophoresis. *Applied and Environmental Microbiology,* 70(8), 4800-4806.

Chapter 3:

Treatment of fish canning wastewater in an aerobic granular reactor[1,2]

Summary

Fish canning effluents, characterized by their high salt content, were treated in a sequencing batch reactor (SBR) where aerobic granular sludge was developed. Mature aerobic granules were obtained after 75 days of operation, with 3.2 mm of diameter, SVI of 30 mL/g TSS and density around 60 g $VSS/L_{granule}$. Applied organic loading rate to the reactor was step-wisely increased until reaching an average value of 3.02 kg $COD_s/(m^3$ d). At this point, the organic matter removal efficiency was of 94%. Ammonia nitrogen was removed via nitrification-denitrification when applied nitrogen loading rates were of 0.18 kg $N/(m^3$ d) with percentages around 38% in terms of nitrogen removal efficiency. Subsequent load increases negatively affected the nitrogen removal efficiency which decreased down to 15% when 0.3 kg $N/(m^3$ d) were applied.

The presence of salt concentrations of 10 g NaCl/L in the treated effluent could be responsible for a delay in the formation of aerobic granules. However it did not cause a detrimental effect on the operation of the reactor once the aerobic granules were formed and only when the salinity of the feeding decreased, changes in the morphology of the granules were observed.

Keywords: aerobic granulation, fisheries, nitrification, nitrogen removal, salt, SBR

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[1] Figueroa M., Mosquera-Corral A., Campos J.L. and Mendez R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. *Water Science and Technology,* **58**(2), 479-485.

[2] Val del Río A., Figueroa M., Arrojo B., Mosquera-Corral A., Campos J.L., García-Torriello G. and Méndez R. (2011). Aerobic granular SBR systems applied to the treatment of industrial effluents. *Journal of Environmental Management.* (accepted)

3.1. INTRODUCTION

The fish canning industry represents one of the main fields in the industrial sector of Galicia and has become an important factor of economic development in coastal areas. Thus, from the approximately 147 Spanish companies that produce canned seafood, 67 are located in Galicia and account for more than 83% of Spanish turnover. Geographically, most of these industries are located in three coastal areas, Ría de Arousa, Ría de Pontevedra and Ría de Vigo.

Like other food processing industries, seafood processing plants and fish canning industries require large quantities of clean water for the preparation and preservation of the final products. This clean water is used to wash, thaw, transport, cook, formulate and package seafood products. The types and concentrations of contaminants generated in the effluents produced in this industry depend on the processed fish species, processing methods and amounts of treated products (Chowdhury *et al.*, 2010). Moreover the use of seawater instead of freshwater in the process is common, thus the generation of wastewater containing large salt concentrations, up to 30 g NaCl/L is possible (Sánchez *et al.*, 2004).

The presence of high salt concentrations in wastewaters affects the physical and biochemical properties of the sludge present in the biological reactors (Lay *et al.*, 2010). Regarding the physical characteristics of the biomass, wastewater containing large salts concentrations was observed to induce high sludge volume indexes (SVI) of the biomass and also to affect the size and dimensions of formed flocs (Moon *et al.*, 2003; Uygur and Kargi, 2004). Furthermore their presence provokes salt stress to the microbial species, which results in the inhibition of many enzymes, decrease of cell activity and eventually leads to plasmolysis (Uygur, 2006).

Nutrients (nitrogen and phosphorous) and organic matter removal from industrial or synthetic wastewater in the presence of NaCl in lab- or full- scale reactors has been researched (Campos *et al.*, 2002; Uygur, 2006; Fontenot *et al.*, 2007; Huiliñir *et al.*, 2008; Rene *et al.*, 2008) and the obtained results draw to similar conclusions. Satisfactory nutrient removal for low salinity wastewaters (0.3 - 2.0 g NaCl/L) has been reported (Intrasungkha *et al.*, 1999), and even a stimulatory effect with enhanced organic carbon removal in a SBR system (Ng *et al.*, 2005) at concentrations below 10 g NaCl/L was observed. Within these ranges of salinity, the acclimation of non-salt adapted micro-organisms to increasing salt concentrations was detected (Campos *et al.*, 2002). However, above 10 g NaCl/L, salt concentrations are supposed to produce considerable osmotic stress and cause the decrease of nitrogen removal efficiencies.

The use of technologies based on aerobic granular sludge could be an alternative for the treatment of wastewater coming from a fish canning industry due to the advantages that the granular biomass presents in terms of the good settling properties, the high biomass

retention and the ability to withstand shock and toxic loadings (Beun *et al.*, 1999; Campos *et al.*, 2009). This technology has been applied to the treatment of low strength municipal wastewater (de Kreuk and van Loosdrecht, 2006) with an organic loading rate (OLR) of 1.2 kg COD/ m^3 d, but also to high strength industrial wastewater, coming from dairy, soybean-processing or malting factories (Arrojo *et al.*, 2004; Schwarzenbeck *et al.*, 2004; Inizan *et al.*, 2005) with OLRs up to 7 kg COD/(m^3 d) and nitrogen loading rates (NLRs) of 0.7 kg N/(m^3 d). Toxic compounds such as phenol, pyridine, chloroanilines, heavy metals or uranium, that can be found in certain wastewaters from coal and refining, pharmaceutical and pesticide processing industries have also been removed in aerobic granular systems (Maszenan *et al.*, 2011).

Recently, the effect of different inlet concentrations of phenol (100-2000 mg phenol/L) on the performance of a granular aerobic reactor seeded with granules containing a mixed phenol degrading consortia acclimatized to salt presence has been studied (Moussavi *et al.*, 2010). In this work, aerobic granules were subjected to increasing salt concentrations up to values of 50 g NaCl/L without loss of the organic matter and phenol removing efficiencies and maintaining the physical properties of the granular biomass.

Although different research studies have been performed to study the effects of different toxic compounds present in industrial wastewater most of them corresponded to the treatment of synthetically prepared wastewater. Further research is needed to evaluate the effect of certain compounds contained in the wastewater itself.

3.2. OBJECTIVE

The main objective of this work was to study the feasibility of the use of an aerobic granular system to treat effluents from a fish canning industry characterized by salinity concentrations up to 10.7 g NaCl/L.

The performance of the system has been evaluated in terms of carbon and nitrogen removal efficiencies and special attention has been paid to the physical properties of the granular biomass.

3.3. MATERIALS AND METHODS

3.3.1. Analytical methods

Ammonia, nitrate, nitrite, pH, volatile suspended solids (VSS), inorganic suspended solids, total suspended solids (TSS) and sludge volume index (SVI) were determined according to the Standard Methods (APHA-AWWA-WPCF, 2005). Inorganic ions (Na^+, Cl^-) were measured by ion chromatography and the salinity, in terms of g NaCl/L, was determined with a conductivity meter (model 524, Crison). Concentrations of total organic carbon (TOC) and inorganic carbon (IC) were measured with an analyser (TOC-5000, Shimadzu). Chemical

Oxygen Demand (COD) was determined by a semi-micro method (Soto *et al*., 1989). Biomass density was determined using dextran blue (Beun *et al.*, 1999). The protein content was determined using a commercial kit (TP0300, Sigma). The morphology and size distribution of the granules were measured regularly by using an image analysis procedure (Tijhuis *et al.*, 1994) with a stereomicroscope (Stemi 2000-C, Zeiss) and by scanning electron microscopy (SEM) (Digital SEM 440, Leica). For SEM analysis the sludge samples were washed with phosphate buffer and fixed with a solution of glutaraldehyde 3% in phosphate buffer overnight. After fixation the sample was dehydrated using ethanol solutions with increasing ethanol concentrations (30, 50, 70 and 100 %).

Respirometric batch assays were performed in a bench-top oxygen meter (BOM5300, Ysi) in order to determine the maximum ammonia oxidizing and heterotrophic activities. A sample containing aerobic granules was taken from the reactor. It was gently washed with phosphate buffer and transferred into vials of 10 mL. Two different fractions were selected; the first one was composed by granules with a mean feret diameter of 7 mm, and the second one by granules with a mean feret diameter of 0.9 mm. The experimental procedure has been reported by Lopez-Fiuza *et al.*, (2002) and described in Chapter 2.

3.3.2. Reactor description

A sequencing batch reactor (SBR) with a working volume of 1.5 L and a height to diameter ratio (H/D) of 5.5 was used (Figure 3.1). Dissolved oxygen was supplied during the aeration phase by a diaphragm pump (Laboport N86, KNF) by using air spargers to promote the formation of small bubbles to guarantee the complete mixture and enough oxygen supply for the biological activity. A set of two peristaltic pumps was used to introduce the feeding solution (on top of the reactor) and to discharge the effluent (at medium height in the column reactor), respectively. The exchange volume was fixed at 50%. A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations of the pumps and valves and regulated the different periods of the operational cycle.

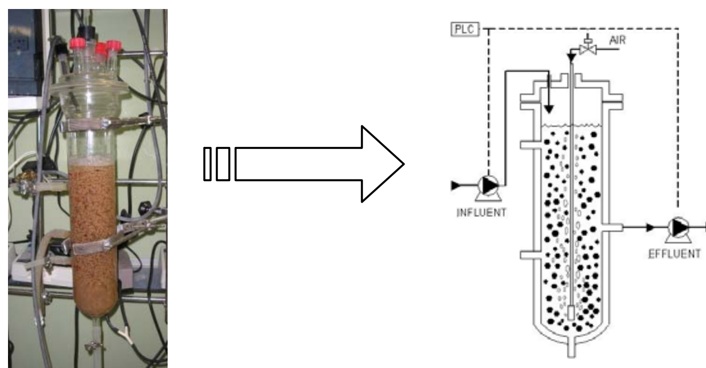


Figure 3.1. Experimental set-up of the SBR: image and diagram of the reactor.

3.3.3. Inoculum

The sludge used as inoculum was a flocculent activated sludge, collected from a nitrifying-denitrifying system operated at a WWTP, which presented a fluffy, irregular and loose morphology as it was observed under the microscope. Physical properties of this sludge were: SVI of 100 mL/g TSS, and settling velocity of 0.25 m/h.

3.3.4. Operational conditions

The SBR was operated in cycles of 3 hours distributed as follows during start up/stable operation: 169/171 minutes of aeration, 3/1 minute of settling, 5 minutes of effluent withdrawal and 3 minutes of feeding.

The feeding supplied to the reactor was prepared using the effluent from an anaerobic digester treating the wastewater produced in a fish canning factory and stored at 4 °C to avoid degradation processes. From day 346 of operation on a small tank with a volume of 750 mL was installed between the refrigeration unit and the reactor in order to keep at room temperature the feeding just one cycle before being added to the reactor. The reactor was operated in five different stages depending on the wastewater composition (Table 3.1.).

Table 3.1. Composition of the feeding media of the SBR.

Stage (dilution)	**S. I (1:10)**	**S. II (1:45)**	**S. III (1:45)**	**S. IV (1:20)**	**S. V (1:10-1:20)**
Parameter *Days*	*0-59*	*60-148*	*149-214*	*215-375*	*376-475*
COD_T (mg O_2/L)	480 ± 50	490 ± 50	440 ±50	771 ± 130	850 ± 278
COD_S (mg O_2/L)	430 ± 57	360 ± 31	326 ± 53	654 ± 129	739 ± 280
TOC (mg TOC/L)	140 ± 21	116 ± 15	108 ± 18	230 ± 47	268 ± 100
OLR (kg COD_s/(m^3 d))	1.72 ± 0.20	1.46 ± 0.10	1.30 ± 0.20	2.92 ± 0.60	3.02 ± 1.24
NH_4^+ (mg NH_4^+-N/L)	62.7 ± 7.0	44.3 ± 8.0	48.8 ± 6.0	83.0 ± 17.0	134.5 ± 55.0
NLR (kg NH_4^+-N/(m^3 d))	0.25 ± 0.03	0.19 ± 0.02	0.20 ± 0.03	0.35 ± 0.07	0.53 ± 0.26
COD/N	6.8	8.1	6.7	7.8	5.5
TSS (mg TSS/L)	200 ± 5	28 ± 3	16 ± 4	14 ± 3	15 ± 3
VSS (mg VSS/L)	70 ± 9	18 ± 1	10 ± 3	8 ± 3	9 ± 3
Conductivity (mS/cm)	14.3 ± 2.0	16.8 ± 0.8	0.12 ± 0.01	0.18 ± 0.02	11.4 ± 2.0
Salinity (g NaCl/L)	9.0 ± 1.2	10.7 ± 0.5	0.06 ± 0.01	0.10 ± 0.01	5.8 ± 0.9
pH	7.1 ± 0.1	7.2 ± 0.3	7.6 ± 0.1	6.9 ± 0.3	6.9 ± 0.3

Different dilution ratios with tap water were applied to the effluent to reach the desired OLR which was in the range 1.3-1.7 kg COD/(m^3 d) in Stages I to III. Due to a modification in

the industry process, during Stages III and IV, the salinity in the feeding was lower than in the previous stages. At the end of this study, in Stage V, the salinity increased up to values of 5.8 g NaCl/L. Moreover, during this last stage the adaptability of the system to changes in the feeding concentration was tested.

3.4. RESULTS AND DISCUSSION

3.4.1. Granule formation and properties

The reactor was operated during 475 days in the operational stages indicated in Table 3.1. During the first ten days of the start up the duration of the settling period was fixed in 3 minutes. Afterwards this time was reduced to 1 minute to promote the washout of poor settling biomass and enhance the aerobic granulation with the minimum settling velocity as driving force of the process (Wang *et al.*, 2004). During the start up, particles with a minimum settling velocity higher than 2.6 m/h were retained in the reactor, later only those particles with settling velocities higher than 8 m/h were able to remain inside the system.

During the start up of the SBR fluffy and elongated biomass structures were formed instead of the typical round shaped granules (Figure 3.2a). This evolution was presumably due to a combination of the difficulty of the removal of the flocculent biomass within the effluent and to an episode of lack of oxygen in the system on day 15 of operation. These elongated biomass structures were not stable and broke up into small pieces after a few days (Figure 3.2b). Subsequently a large part of the biomass was washed out and a new granulation event occurred. The new formed granules (S. I, day 40) still presented small fibrous structures in their surface that gradually disappeared. If an homogenous round appearance of the biomass is considered to belong to granular sludge, mature granules were obtained only from day 75 on, with an average feret diameter of 3.2 mm (S. II); however, if the sludge volume index (SVI) value below 75 mL/g TSS is considered as an indication of good settling properties of the sludge, aerobic granules were formed already after day 34, when the sludge settling was clearly improved in the reactor and the SVI was below 60 mL/g TSS.

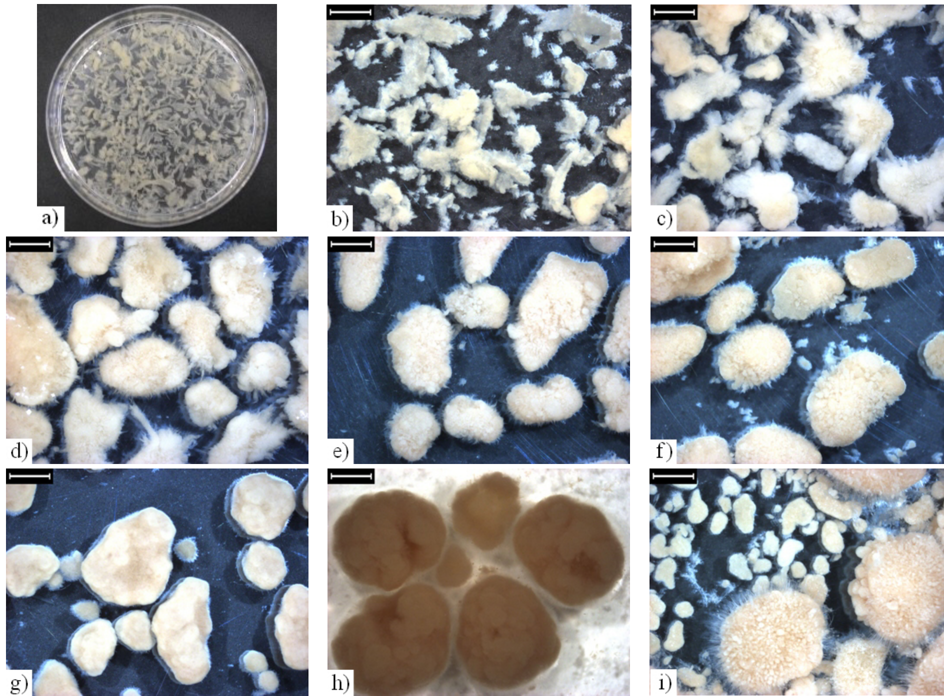


Figure 3.2. Biomass from the SBR reactor on days: a) 22 (S. I), b) 40 (S. I), c) 64 (S. II), d) 75 (S. II), e) 102 (S. II), f) 142 (S. II), g) 208 (S. III), h) 298 (S. IV) and i) 416 (S. V). The bar indicates 2 mm.

During the whole operational period, the size distribution of the granules varied in terms of volume and average feret diameter (Figure 3.4 and Figure 3.3). The average feret diameter gradually increased from days 64 to 142 (S. II). After this date, a decrease in the feret diameter was observed due to the breakage of the bigger granules and the formation of granules with a feret diameter around 1 mm. With the increase of loading rate and the course of time, these granules reached again a size around 6 mm and a new break up event was observed (day 354 on).

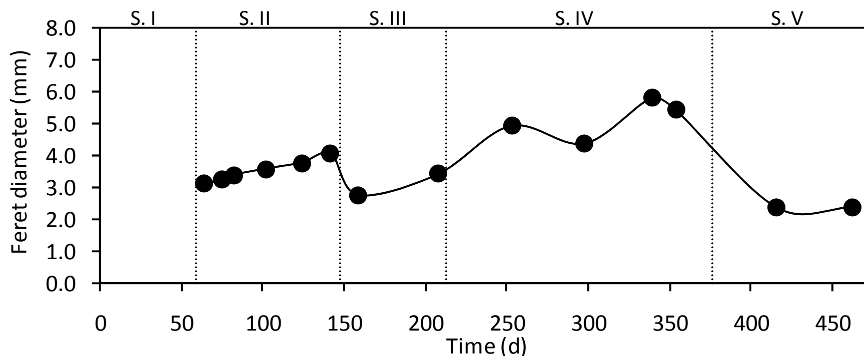


Figure 3.3. Evolution of the average feret diameter of the granules during the operational time.

The evolution of the granular biomass formation and breakage is also reflected in Figure 3.4, where the contribution to the total volume of each fraction of diameter can be observed. The volumetric distribution shows how the granules with large diameters developed during the operational time, with the appearance of bars in the right side of the histogram. Generally, the contribution to the total volume of each fraction of granules with diameter below 2 mm is around 0.2%, but on day 416, due to the high percentage of small granules, the contribution of fractions between 1 and 2 mm is significant and corresponded to a 1.5%.

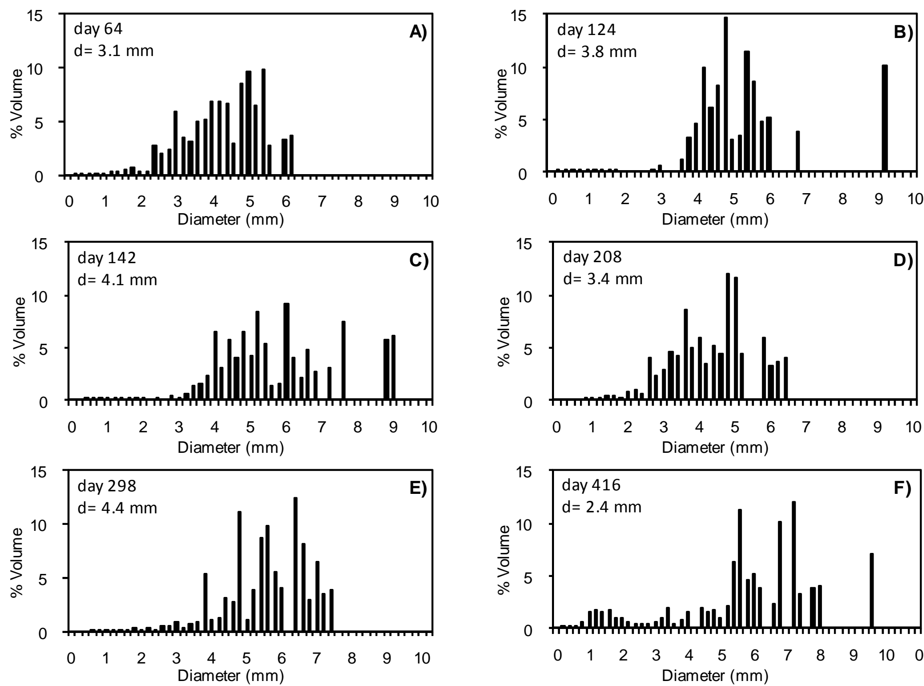


Figure 3.4. Size distribution of granular sludge on operating days 64 (S. II) A), 124 (S. II) B), 142 (S. II) C), 208 (S. III) D), 298 (S. IV) E) and 416 (S. V) F).

During Stage S. V, the presence of two different types of granules was clearly observed (Figure 3.2i; Figure 3.4F). A high volume of the reactor was occupied by granules with a size higher than 6 mm, and when the biomass settled a small fraction of granules with a small feret diameter could be also observed forming a layer on top of the previous ones. The existence of both types of granules could be caused by two reasons: a) the continuous development of new aggregates which grow in size slowly, and b) the disintegration of granules with bigger sizes, where anaerobic zones are formed as consequence of oxygen transfer limitations. Furthermore the specific substrate utilization and specific biomass growth rates are negatively related to the size of granules (Liu *et al.*, 2005b) so to promote the formation of granules of small size is advisable.

The composition characteristics of the treated wastewater led to the formation of granules with a rough surface and an irregular outline when they were observed under the stereomicroscope. Only in stages S. III and S. IV granules with smooth outlines were observed (Figure 3.2). However, the images obtained with SEM on day 374 (S. IV) (Figure 3.5) showed a high abundance of filamentous microorganisms tightly wrapped and connected with the rod-shaped bacteria and cocci. It was previously reported that filamentous bacteria could act as a structural backbone for the first step of the formation of aerobic granules (Beun *et al.*, 1999; Wang *et al.*, 2006), however, in this work the overgrowth of filamentous bacteria, which made granules to have rough surface, was observed along different periods of operation.

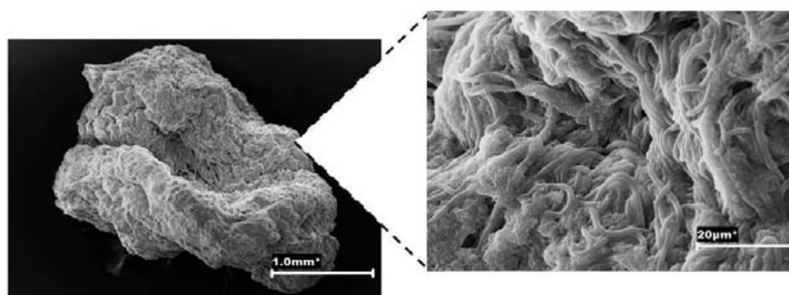


Figure 3.5. SEM images of a granule on day 374 of operation (S.IV).

During stages S. I and S. II the SVI gradually decreased, from values of 82 mL/g TSS to 28 mL/g TSS on day 82, and then it was maintained around 30 mL/g TSS. On day 208 of operation (S. III), the obtained granules presented very good physical properties; they exhibited density values around 60 g $VSS/L_{granule}$ and a SVI of 30 mL/g TSS. During stage S. IV the value of the SVI increased together with the feret diameter of the granules, until reaching a value of 75 mL/g TSS on day 368. With the breakage of aerobic granules in stage S. V, the value of the SVI decreased again down to a value of 20 mL/g TSS. Despite the variability of the SVI values during the operation of the reactor, these values are in accordance with those obtained for other aerobic granular sludge reactors treating industrial wastewater, that were between 30 and 90 mL/g TSS (Arrojo *et al.*, 2004; Schwarzenbeck *et al.*, 2004; Su and Yu, 2005). For example, treating an industrial wastewater from a laboratory for analysis of dairy products, at OLR and NLR of 1 kg COD/(m^3 d) and of 0.1 kg NH_4^+-N/(m^3 d), respectively, granules with densities of 10-15 g $VSS/L_{granule}$ and SVI of 60 mL/g TSS were obtained (Arrojo *et al.*, 2004). The differences in the values of density and SVI obtained in this work, compared to the other reported values, could be related to the kind of treated wastewater.

The formation of aerobic granules during stages S. I and S. II and the increase of solids content inside of the reactor provoked that the sludge retention time reached a value of 60 d in the reactor. The microorganisms growth yield expressed in terms of gram of biomass produced per gram of COD consumed (biomass production) has been calculated during these periods. The maximal biomass production, between days 54 and 82, was of only 0.16 g VSS/g

COD despite the rapid growth of biomass in the system. Even though, this is a value similar to other ones for aerobic granular systems (between 0.20 and 0.33 g VSS/g $COD_{removed}$) (Tay *et al.*, 2001; Liu *et al.*, 2005a; Mosquera-Corral *et al.*, 2005a) and lower than the reported values for activated sludge systems with a typical growth yield of 0.4-0.6 g VSS/g $COD_{removed}$ (Droste *et al.*, 1997).

The solids concentrations in the reactor increased up to 15 g VSS/L at the end of this study while the biomass concentrations in the effluent ranged between 0.05 and 0.35 g TSS/L during the different operational stages (Figure 3.6). The highest concentrations of VSS and TSS in the effluent were caused by natural purges of biomass from the reactor, when the granules reached the level of the withdrawal port due to the biomass accumulation inside the reactor.

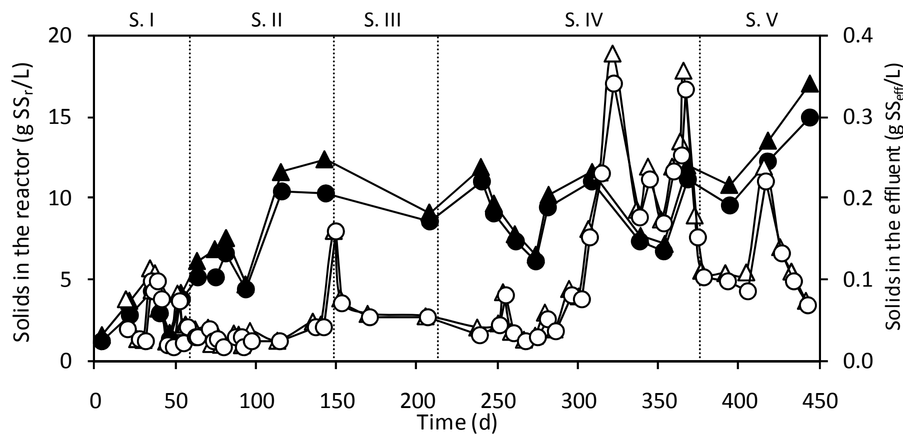


Figure 3.6. Solids content in the reactor: TSS (▲) and VSS (●); and in the effluent TSS (△) and VSS (○) (g SS/L).

Considering the estimated maximal growth yield of the aerobic granules (0.16 g VSS/g COD), a mass balance between days 150 and 280 was calculated. The amount of solids washed out from the system in that period, considering 2 days with a purge of 0.16 g VSS/l, was of 20.7 g VSS and the theoretical amount of produced biomass from all the consumed COD was of 16.7 g VSS. This balance indicated that the amount of biomass in the system should decrease around 2.7 g VSS/L, which is in the range of the observed 3.7 g VSS/L. Therefore the amount of produced biomass is well balanced with the amount of biomass washed out. In order to fulfill the effluent standards in terms of solids content when this episodes of solids washout are produce, it would be necessary to establish a post-treatment step, e.g. a lamella settler as proposed by de Bruin *et al.*, (2004).

The presence of NaCl and other salts in the feeding could affect the inorganic to total suspended solids ratio which ranged, in the present work, from 35 to 65%. Moreover, the formation of precipitates due to the salt content of the influent, could contribute to the

retention of the flocculent biomass inside the reactor. Similar observations were made in nitrifying systems treating high salt concentrated wastewater (Mosquera-Corral *et al.*, 2005b).

Finally, the main physical parameters of the obtained granules and their evolution in a specific day of each period are resumed in Table 3.2.

Table 3.2. Main physical parameters in different operational days

Stage (Dilution)	**S. I (1:10)**	**S. II (1:45)**	**S. III (1:45)**	**S. IV (1:20)**	**S. V (1:10-1:20)**
Parameter *Day*	*54*	*143*	*208*	*368*	*471*
SVI (mL/g TSS)	53	34	30	75	20
Density (g VSS/$L_{granule}$)	45	66	60	29	68
Diameter (mm)	3.0	4.0	3.3	5.5	2.4
VSS_r (g/L)	3.78	16.65	8.64	11.20	15.20
VSS_{eff} (g/L)	0.073	0.042	0.053	0.330	0.330

3.4.2. Effect of the salt content

Regarding to the salt content, high concentrations of this compound are known to compromise the correct operation of conventional aerobic wastewater treatment processes since they produce high osmotic pressure on bacterial cells, which causes plasmolysis, dehydration and disintegration of the bacteria cell and loss of the cell activity. The presence of salinity also increases the viscosity of water and correspondingly modifies the mass transfer rates of substrates by the decrease of diffusivity rates in the boundary layer (Hill, 2009). However, the treatment of wastewater containing salt concentrations up to 60 g NaCl/L was feasible in SBRs and membrane bioreactors (Uygur and Kargi, 2004; Abou-Elela *et al.*, 2010; Lay *et al.*, 2010).

Different studies have been performed using synthetic wastewater in order to test the effects of the increase of salts content on the organic matter, nitrogen and phosphorous removal efficiencies in activated sludge systems. In many cases, authors established correlations between the salt content and the decrease of nutrient removal efficiencies (Sánchez *et al.*, 2004; Uygur and Kargi, 2004; Ng *et al.*, 2005). For example, Uygur and Kargi, (2004) observed that the drop of the COD removal efficiency was of 5% and 10.5% when treating 5 g NaCl/L and 10 g NaCl/L, respectively. The nitrification process is also affected by high concentrations of sodium chloride which reduce the rates of both steps although nitratation was found to be the most sensitive step (Sánchez *et al.*, 2004); therefore the accumulation of nitrite is also a fact when treating high salinity wastewaters. In this case, using the proposed correlation for the decrease of the nitrite oxidation rate, a theoretical

drop around 7% and 14% when treating 5 g NaCl/L and 10 g NaCl/L, respectively, was predicted.

The effect of the increase of salinity in the morphology of flocs has also been studied (Moon *et al.*, 2003; Mesquita *et al.*, 2009). In this case, the average floc size and fractal dimension decreased at a salt concentration up to 10 g NaCl/L due to floc disruption. This fractal dimension of the floc relates the mass with the size of the floc, therefore a high value of the fractal dimension corresponds to a compact aggregated floc and a low value corresponds to the loose aggregated floc. It has also been observed that as the salt concentration increased, the SVI increased due to the deteriorated settling properties of sludge (Moon *et al.*, 2003; Uygur and Kargi, 2004; Ng *et al.*, 2005), e.g. from 50 mL/g TSS at 5 g NaCl/L to nearly 97 mL/g TSS at 60 g NaCl/L in activated sludge systems (Uygur and Kargi, 2004). Visual macroscopic observations also revealed changes in some microbial populations. Protozoa and rotifers were removed from the sludge when the NaCl concentrations increased. Ciliates became the dominant micro-organisms at NaCl concentrations of 5 g NaCl/L but they were absent when the concentration was increased beyond 10 g NaCl/L (Ng *et al.*, 2005).

In the present work, appreciable changes of the organic matter and nitrogen removal efficiencies were not observed when the salinity of the wastewater was reduced in stage S. III and increased in S. V (see next section). This fact can be explained as a result of the microbial acclimation during previous stages. On the one hand, an improvement of the organic matter removal when treating synthetic wastewater at concentrations up to 10 g NaCl/L was observed (Ng *et al.*, 2005), so it would be expected a certain decrease of removal efficiency in S. III. On the other hand, some authors have observed that the increase of salt concentration did not affect the removal efficiency as a result of acclimation of the biomass when the system was operated with increasing concentrations of salinity due to the presence of some adapted halophilic organisms in the inoculum (Kargi and Uygur, 1997; Intrasungkha *et al.*, 1999; Campos *et al.*, 2002). In this way the microorganisms could easily adapt to changes in salinity of the feeding media.

The decrease of salinity did not affect the SVI either, but could cause the disappearance of the filamentous structures on the surface of the granules (Figure 3.7). This could be explained by the new growth of protozoa and rotifers that graze on certain suspended organisms and also probably on those filamentous structures observed on stages S. II and S. V (Nicolau *et al.*, 2001; Weber *et al.*, 2007).

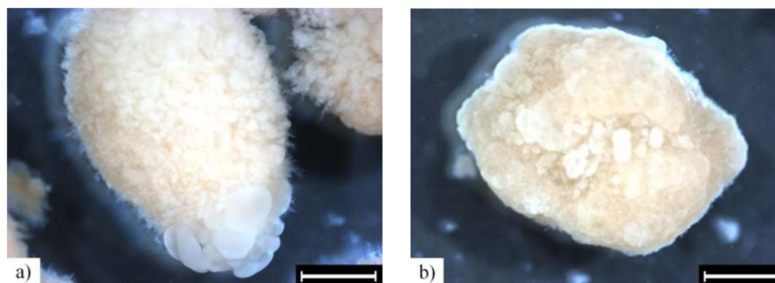


Figure 3.7. Morphology of aerobic granules after the reduction of salinity, on days A) 142 (S. II with 10.7 g NaCl/L) and B) 159 (S. III with 0.05 g NaCl/L). The bar represents 2 mm.

3.4.3. Organic matter and nitrogen removal

The organic matter removal efficiency, expressed in terms of COD, was between 85 and 94% during the whole operational period. Data related to organic matter content and removal efficiencies are shown in Figure 3.8.

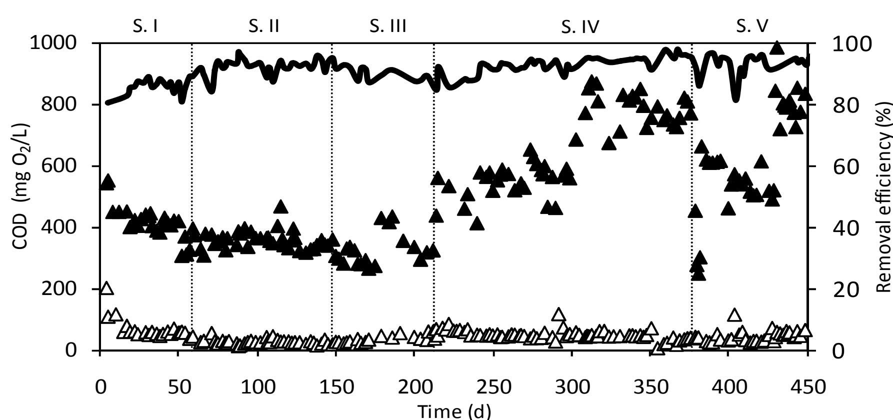


Figure 3.8. Organic carbon concentration in the influent (COD_{inf}, ▲), effluent (COD_{eff}, △) (mg O_2/L) and removal efficiency percentages (—) (%).

The system could withstand the increase in the OLR in the different operational stages with maintaining high removal efficiencies (Table 3.3). Lower values of removal efficiency were detected only during the first days of operation whereas the biomass was adapting and growing under the new operational conditions and also when fluctuating OLR were promoted during stage S. V. In this case, the system could easily recover previous removal efficiencies.

Table 3.3. Organic matter content in the effluent and removal efficiencies during the different operational stages.

		Stage Dilution	S. I (1:10)	S. II (1:45)	S. III (1:45)	S. IV (1:20)	S. V (1:10-1:20)
	Parameter	*Day*	*0-59*	*60-148*	*149-214*	*215-375*	*376-475*
Inf.	TOC (mg TOC/L)		140 ± 21	116 ± 15	108 ± 18	230 ± 47	268 ± 100
Inf.	OLR (kg $COD_s/(m^3$ d))		1.72 ± 0.20	1.46 ± 0.10	1.30 ± 0.20	2.92 ± 0.60	3.02 ± 1.24
Eff.	TOC (mg TOC/L)		22 ± 11	9 ± 2	11 ± 4	16 ± 5	16 ± 6
Eff.	COD_S (mg COD/L)		67 ± 34	26 ± 8	34 ± 12	48 ± 15	46 ± 18
Eff.	OLR (kg $COD_s/(m^3$ d))		0.25 ± 0.08	0.11 ± 0.04	0.14 ± 0.05	0.19 ± 0.06	0.19 ± 0.08
$OLR_{treated}$ (kg $COD_s/(m^3$ d))			1.47 ± 0.18	1.35 ± 0.14	1.15 ± 0.19	2.73 ± 0.55	2.83 ± 1.11
Removal efficiency (%)			85 ± 5	92 ± 2	88 ± 2	93 ± 3	93 ± 5

The treated OLR is in the range of the one achieved with other industrial wastewater used as influent of aerobic granular reactors. For example, a value of 3.2 kg $COD_s/(m^3$ d) was reached with malting wastewater (Schwarzenbeck *et al.*, 2004), 3.5 kg $COD_s/(m^3$ d) with brewery wastewater (Wang *et al.*, 2007) or 2.7 kg $COD_s/(m^3$ d) with slaughterhouse wastewater (Yilmaz *et al.*, 2008). However, higher treated OLR are possible in aerobic granular reactors. Values around 7 kg $COD_s/(m^3$ d) have been reported for the treatment of wastewater from a laboratory for analysis of dairy products (Arrojo *et al.*, 2004) or soybean processing effluents (Su and Yu, 2005).

The nitrogen removal presented a distinct pattern during the different operational stages. During stage S. I, the ammonia concentration in the effluent was 15 mg NH_4^+-N/L higher than in the influent probably due to the hydrolysis in the reactor of nitrogenous compounds such as proteins contained in the wastewater. Values between 50 and 60 mg Protein/L were measured in the influent while the values in the effluent were under the detection limit. If the nitrogen content in the proteins of 16% is considered, the amount of released nitrogen would be around 10 mg N/L. Since the biomass would also use part of the ammonia for growth, the differences in the ammonia content between the influent and effluent should be explained by the presence of other nitrogenous compounds in the feeding that released nitrogen.

During stage S. II, the carbon to nitrogen (COD/N) ratio increased due to the change in the wastewater composition, and, after ten days, values of nitrite concentrations around 30 mg NO_2^--N/L were achieved in the effluent, with nitrate concentrations below 4 mg NO_3^--N/L (Figure 3.9). The oxidation of ammonia mainly to nitrite was maintained during stages S. II and S. III, with ammonia removal efficiencies around 90%, whereas nitrogen removal efficiencies ranged between 23 and 45 % in both stages.

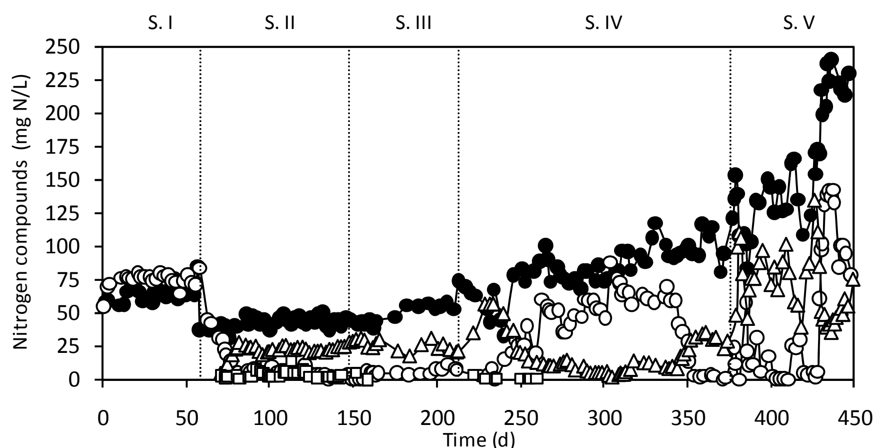


Figure 3.9. Ammonia concentration in the influent (●) and ammonia (○), nitrite (△) and nitrate (□) concentrations in the effluent (mg N/L).

During stage S. IV, from day 264 to 350, a drastic reduction in nitritation was observed, with ammonia removal efficiencies that ranged from 10 to 40%. During this period the inorganic carbon concentration (carbonate and bicarbonate) in the feeding decreased due to the use of a batch of feeding media with 15 mg IC/L. It seems that alkalinity was a limiting factor, because from day 350 on, and coinciding with the addition of a new bicarbonate rich-feeding medium with 100 mg IC/L, nitrite was measured again in the bulk liquid.

Stage S. V was characterized by the increase of the ammonia concentration, from 125 up to 230 mg NH_4^+ -N/L. During this stage, nitrogen removal was highly variable, with short periods with nitrite concentrations that ranged between 35 and 130 mg NO_2^--N/L.

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 efficiencies achieved by both mechanisms, a nitrogen mass balance was made to the reactor (Table 3.4) during stages S. II (from day 80 to 149), S.III (from day 150 to 212), S. IV (from day 260 to 345) and S. V (from day 384 to 428), following the procedure reported in Chapter 2.

Even though the nitrogen removal efficiency of the system in the analyzed periods is similar, the results from the balance indicated that the percentage of nitrogen denitrified referred to the total nitrogen removed was of around 78% in S. II, 64% in S.III, 33% in S. IV and 69% in S. V (Table 3.4). Percentages of assimilation are similar between the selected periods of S. II, S. III and S.V. In spite of similar nitrogen removal efficiencies between S. II and S.IV the nitrogen fraction that was assimilated and denitrified was different. The high percentage of nitrogen assimilated during stage S. IV was caused by the unbalanced growth of biomass due to the increase of OLR from 1.30 to 2.92 kg COD/(m^3 d).

Table 3.4. Nitrogen mass balances during different operational stages.

Days	$N_{removed}$ [a] (mg N/d)	$N_{assimilated}$ [b] (mg N/d)	Nitrogen removal efficiency (%)		
80-149 (S. II)	77.4	16.9	29	$N_{assimilated}$ (%)	22
				$N_{denitrified}$ (%)	78
150-212 (S. III)	132.0	47.6	40	$N_{assimilated}$ (%)	36
				$N_{denitrified}$ (%)	64
260-345 (S. IV)	135.0	90.8	26	$N_{assimilated}$ (%)	67
				$N_{denitrified}$ (%)	33
386-428 (S. V)	256.2	79.4	32	$N_{assimilated}$ (%)	31
				$N_{denitrified}$ (%)	69

[a]Calculated as the difference between the influent and the effluent nitrogen content, [b]calculated from the biomass growth.

The occurrence of nitrification and denitrification was checked by means of monitoring the substrate profiles in different operational cycles during the operation of the reactor. Concentrations of the carbon and nitrogen compounds were tracked in the liquid phase in cycles from day 148 (period between days 80 to 149), day 270 (period between days 260 to 345) and day 396 (period between days 386 to 428) (Figure 3.10).

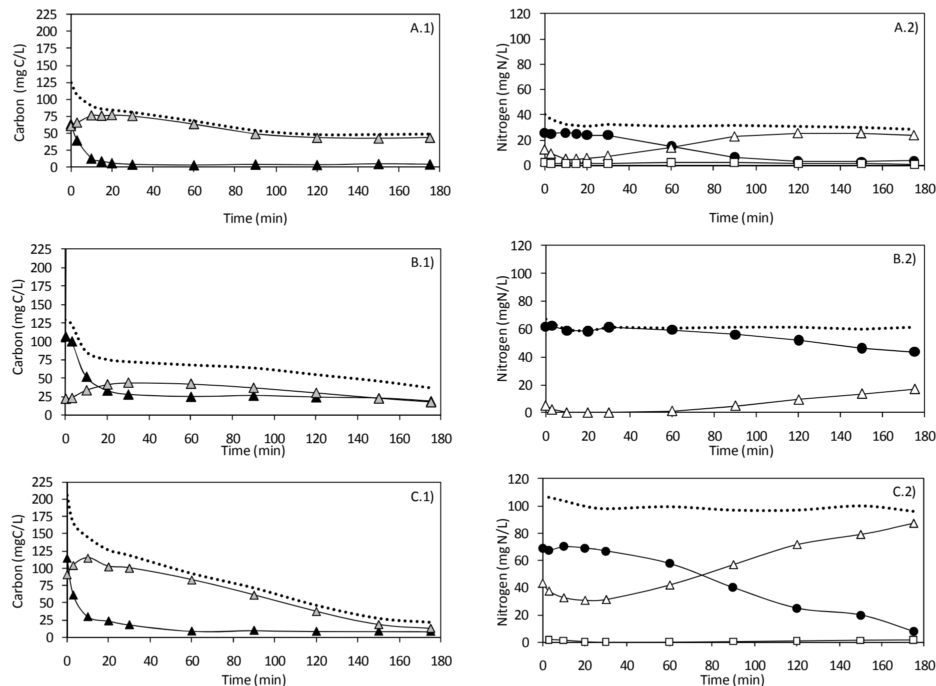


Figure 3.10. 1) IC (▲), TOC (▲) and TC (…). 2) NH_4^+-N (●),NO_2^--N (△), NO_3^--N (□) and TN (…) concentrations (mg/L). Cycles performed on operational days A) 148 (S. II), B) 270 (S. IV) and C) 396 (S. V).

In all the cases the biodegradable COD was almost fully removed at the beginning of the cycle (feast phase) although the length of this phase slightly increased with the increase of the OLR. From day 148 to 396 the length of the feast phase increased from 20 to more than 30 minutes. The high variability in the nitrogen removal that is shown in Figure 3.9 is also reflected in the obtained profiles of different days of operation (Figure 3.10). In all the cases it can be observed that denitrification takes place in the first minutes of the cycle (when nitrite or nitrate and organic matter were present) and also that the concentration of nitrogen removed was around 10 mg N/L (or less) per cycle. The nitrogen assimilated for growth is supposed to be higher during the feast period compared to the famine period due to the higher COD removal, but in these cases the effect is not clearly observed.

Ammonia was oxidised to nitrite during the aerobic period immediately after the disappearance of biodegradable COD from the liquid phase. From this point on, the maximal ammonia consumption rate (Mosquera-Corral *et al.*, 2005a) and other parameters were calculated in each cycle and they are shown in Table 3.5.

Table 3.5. Summary of the results obtained in each one of the different cycles.

Parameter Stage	Day 148 S. II	Day 270 S. IV	Day 396 S. V
DO concentration (feast/famine)	6.0/8.0	5.5/8.3	5.2/6.9
Biomass concentration (g VSS/L)	10.30	6.10	10.01
Removal efficiency of nitrogen (%)	29	10	9
Removal efficiency of ammonia (%)	91	29	90
Maximal ammonia specific consumption rate (mg NH_4^+-N/(g VSS d))	40.3	34.5	67.2
Maximal nitrite specific production rate (mg NO_2^--N/(g VSS d))	35.7	32.2	56.6
Nitrogen removed during the cycle (mg N/L)	11.8	5.2	9.9
Ammonia consumed (mg NH_4^+ -N/L)	22.1	18.1	61.2
Nitrite and nitrate produced (mg N/L)	20.4	17.9	57.5
COD removed (mg COD/L)	175.1	262.4	318.2
Nitrogen used for growth (mg N/L)*	4.0	5.9	7.2
COD/N ratio	7.5	7.8	5.6

*Supposing 0.19 g VSS/g COD and 0.12 g N/g VSS

The differences observed in the selected operational days are reflected in the table. It can be observed that on the three selected days practically all the ammonia was oxidized into nitrite (and nitrate with concentrations below 2 mg NO_3^--N/L) although the ammonia removal efficiency was different between days 148, 396 and day 270. Therefore, final products obtained in day 270 were 43 mg NH_4^+-N/L and 17 mg NO_2^--N/L and on day 396 they were 7.9

mg NH_4^+ -N/L and 87 mg NO_2^--N/L. The high DO concentration in the bulk liquid in the cycle performed on day 396 and the lack of enough anoxic zones in the granule could cause that the amount of nitrite denitrified were only around 13 mg NO_2^--N/L.

The amount of nitrogen consumed for growth in each cycle was calculated from the COD removed in the cycle in order to minimize the experimental errors associated with the solids concentration determination at the beginning and at the end of the cycle. The obtained values matched with the total nitrogen concentration that was eliminated.

Maximal ammonia and nitrite consumption rates are similar between days 148 and 270, and higher on day 396. These differences could be explained by different limiting factors that have to be considered in biological nitrogen processes: alkalinity limitations, temperature, substrate diffusion and transport, substrate inhibition, or dissolved oxygen concentration (Fdz-Polanco *et al.*, 1994; Zhu and Chen, 2002; Vázquez-Padín *et al.*, 2010a) that could limit the ammonia oxidation. These factors were considered for the interpretation of the different profiles measured and to correlate with the data measured during the operation of the reactor.

On day 274 (S. IV) the effect of dissolved oxygen on the nitrification process was tested. A cycle measurement was performed on day 270 with the usual dissolved oxygen (DO) concentration (value around 8 mg O_2/L in the famine phase); on day 274, the system was aerated with pure oxygen, and the value of DO was kept around 30 mg O_2/L. The calculated maximal ammonia consumption rate was of 59 mg N/(g VSS d), higher than that obtained on day 270, and the overall ammonia removal increased up to 48% (Figure 3.11).

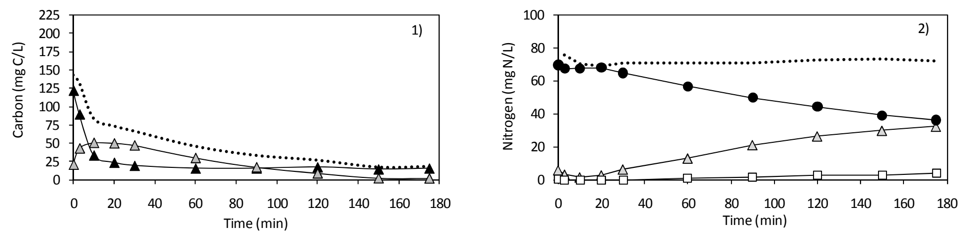


Figure 3.11. A) IC (▲), TOC (▲) and TC (…); and B) NH_4^+-N (●),NO_2^--N (△), NO_3^--N (□) and TN (…) concentrations (mg/L). Cycle performed on day 274 (S. IV) with 30 mg/L of dissolved oxygen.

The increase of DO concentration promoted the total consumption of the inorganic carbon (IC) and this could cause a limitation in the ammonia oxidation, as previously reported by Guisasola *et al.* (2007). Enough alkalinity is necessary to carry out nitrification and even though the organic matter oxidation released bicarbonate it would be necessary to provide a sufficient amount of this compound for the biochemical reaction in case of the oxygen diffusion limitation were avoided.

3.4.4. Respirometric assays

Respirometric assays were performed on day 381 (S. V) in order to test the nitrifying activity of the granules corresponding to both differentiated groups of granules as they were observed from the settled biomass. Both groups were defined according to their respective contribution to the biomass volume either due to their small size or to the low number of particles. The group of granules which contributed the less, named small granules, were characterized by diameters from 0.6 to 2.4 mm while in the group of the big granules were included those with diameters between 2.6 and 9.6 mm (Figure 3.12). The obtained average diameters were of 0.9 and 7.0 mm for the groups of small and big granules, respectively.

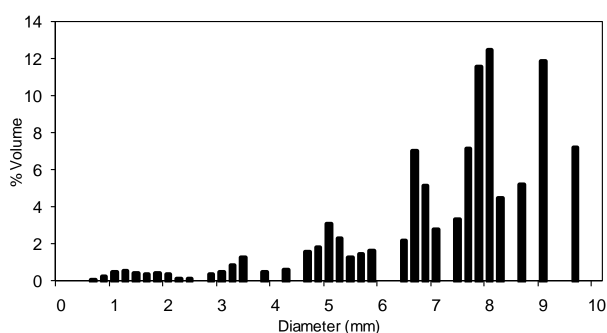


Figure 3.12. Volumetric size distribution (%) on day 381.

Obtained maximum specific ammonia oxidizing activity of the small and big granules was of 178 mg NH_4^+-N/(g VSS d) and 42 mg NH_4^+-N/(g VSS d), respectively (Figure 3.13). On this operational day, the activity of the reactor was estimated, having into account that 150 mg NH_4^+-N/L were consumed in one cycle (3 h) and that the concentration of solids inside the reactor was around 9 g VSS/L, as 66 mg NH_4^+-N/(g VSS d).

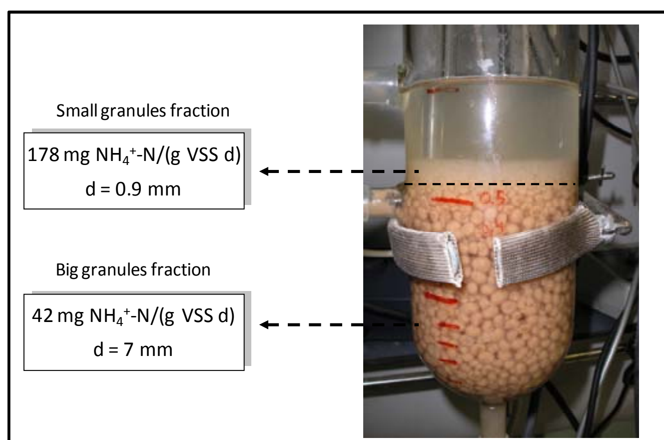


Figure 3.13. Image of both granules fractions together with their corresponding ammonia oxidizing activities.

In order to evaluate the share of biomass present in each granules fraction and contributing to the global nitrifying activity a calculation was performed. Initially the weighting of the specific ammonia oxidizing activity was calculated considering the two size fractions of granules and the average ammonia oxidizing activity in the system. The biomass fraction in percentage, of each type of granules that contributes to the overall ammonia oxidizing activity was estimated as 18% and 82% for the small and big granules, respectively. With these data the contribution to the ammonia oxidizing activity of each fraction was determined as 48 and 52% for small and big granules, respectively. This result indicated that in between the big granules a large amount of biomass is not active.

From the size distribution of the granules on that day of operation, the total area of the selected sizes was calculated (extrapolated from a representative sample from the reactor) and also the area corresponding to the two fractions. In this case, the contribution to the total area of the smallest granules accounted for the 20%. This is of importance due to the fact that this area corresponds to the exchange surface between the different substrates and the biomass granules. Therefore, even though the volume occupied by the biggest aerobic granules is the highest, around 90%, an important part of the aerobic area is associated with the smallest granules, and the biggest granules could be then responsible of anoxic or even anaerobic reactions.

The differences in the obtained activities corresponding to both different size granules are related to external and internal mass transfer limitations. Some mathematical modeling and experimental work has been carried out to research the mass transfer within granules (Gapes *et al.*, 2004; Liu *et al.*, 2005b; Gapes and Keller, 2009; Vázquez-Padín *et al.*, 2010b). The impact of external mass transfer causes a great difference in DO concentrations between the bulk liquid and the aggregate surface (Wilen *et al.*, 2004), but also internal mass transfer provides a significant rate limitation. As particle size increases, the importance of mass transfer limitations increases significantly (Gapes *et al.*, 2004).

3.5. CONCLUSIONS

The start up of a SBR to obtain aerobic granular sludge was slower compared to other aerobic granular systems when wastewater from a fish canning industry, characterized by high salt content, was treated. After 75 days of operation stable granules were obtained with the following physical properties: 3.4 mm of average diameter, SVI of 30 mL/g TSS and density around 60 g VSS/$L_{granule}$.

The use of a SBR system with aerobic granular biomass allowed withstanding the variations in the saline conditions of the treated wastewater that varied between 0.06 and 10.7 g NaCl/L. The decrease of salinity did not affect the SVI, but could cause the disappearance of the filamentous structures on the surface of the granules.

The maintenance of high concentrations of biomass, between 10 to 15 g VSS/L, with good settling properties allowed the treatment of OLR up to 2.8 kg COD/(m^3 d) at the end of the operational period of the reactor. Organic matter removal efficiencies were over 85%, and nitrification-denitrification was also developed in the system. The ammonia was mainly oxidized to nitrite and the efficiency of nitrogen removal was limited to values between 26 and 40% of the nitrogen in the influent. Oxygen transfer limitation was found to affect the performance of the nitrification process in the reactor.

In order to increase the removal efficiency of aerobic processes is recommendable to avoid the development of granules with a big diameter, since they occupied most of the biomass volume with a specific activity similar to the obtained from small granules which occupied around 10% of the volume.

3.6. REFERENCES

Abou-Elela S.I., Kamel M.M. and Fawzy M.E. (2010). Biological treatment of saline wastewater using a salt-tolerant microorganism. *Desalination,* 250(1), 1-5.

APHA-AWWA-WPCF (2005) Standard methods for the examination of water and wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Arrojo B., Mosquera-Corral A., Garrido J.M. and Mendez R. (2004). Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research,* 38(14-15), 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.

Campos J.L., Mosquera-Corral A., Sanchez M., Mendez R. and Lema J.M. (2002). Nitrification in saline wastewater with high ammonia concentration in an activated sludge unit. *Water Research,* 36(10), 2555-2560.

Campos J.L., Figueroa M., Mosquera-Corral A. and Méndez R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering,* 1(2), 136-151.

Chowdhury P., Viraraghavan T. and Srinivasan A. (2010). Biological treatment processes for fish processing wastewater - A review. *Bioresource Technology,* 101(2), 439-449.

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: an alternative to activated sludge? *Water Science and Technology,* 49(11-12), 1-7.

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

Droste R.L., Wiley and Sons (1997) Theory and practice of water and wastewater treatment, Wiley & Sons, New York.

Fdz-Polanco F., Villaverde S. and Garcia P.A. (1994). Temperature effect on nitrifying bacteria activity in biofilters: Activation and free ammonia inhibition. *Water Science and Technology,* 30(11), 121-130.

Fontenot Q., Bonvillain C., Kilgen M. and Boopathy R. (2007). Effects of temperature, salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. *Bioresource Technology,* 98(9), 1700-1703.

Gapes D., Wilen B.M. and Keller J. (2004). Mass transfer impacts in flocculent and granular biomass from SBR systems. *Water Science and Technology,* 50(10), 203-212.

Gapes D.J. and Keller J. (2009). Impact of oxygen mass transfer on nitrification reactions in suspended carrier reactor biofilms. *Process Biochemistry,* 44(1), 43-53.

Guisasola A., Petzet S., Baeza J.A., Carrera J. and Lafuente J. (2007). Inorganic carbon limitations on nitrification: Experimental assessment and modelling. *Water Research,* 41(2), 277-286.

Hill G.A. (2009). Oxygen mass transfer correlations for pure and salt water in a well-mixed vessel. *Industrial & Engineering Chemistry Research,* 48(7), 3696-3699.

Huiliñir C., Cristina Martí M., Aspé E. and Roeckel M. (2008). Organic and nitrogenous matter effects on the denitrification of saline and protein-rich effluents. *Environmental Technology,* 29(8), 881 - 890.

Inizan M., Freval A., Cigana J. and Meinhold J. (2005). Aerobic granulation in a sequencing batch reactor (SBR) for industrial wastewater treatment. *Water Science and Technology,* 52(10-11), 335-343.

Intrasungkha N., Keller J. and Blackall L.L. (1999). Biological nutrient removal efficiency in treatment of saline wastewater. *Water Science and Technology,* 39, 183-190.

Kargi F. and Uygur A. (1997). Biological treatment of saline wastewater in a rotating biodisc contactor by using halophilic organisms. *Bioprocess and Biosystems Engineering,* 17(2), 81-85.

Lay W.C.L., Liu Y. and Fane A.G. (2010). Impacts of salinity on the performance of high retention membrane bioreactors for water reclamation: A review. *Water Research,* 44(1), 21-40.

Liu L.L., Wang Z.P., Yao J., Sun X.J. and Cai W.M. (2005a). Investigation on the properties and kinetics of glucose-fed aerobic granular sludge. *Enzyme and Microbial Technology,* 36(2-3), 307-313.

Liu Y.Q., Liu Y. and Tay J.H. (2005b). Relationship between size and mass transfer resistance in aerobic granules. *Letters in Applied Microbiology,* 40(5), 312-315.

Lopez-Fiuza J., Buys B., Mosquera-Corral A., Omil F. and Mendez R. (2002). Toxic effects exerted on methanogenic, nitrifying and denitrifying bacteria by chemicals used in a milk analysis laboratory. *Enzyme and Microbial Technology,* 31(7), 976-985.

Maszenan A.M., Liu Y. and Ng W.J. (2011). Bioremediation of wastewaters with recalcitrant organic compounds and metals by aerobic granules. *Biotechnology Advances,* 29(1), 111-123.

Mesquita D.P., Amaral A.L., Ferreira E.C. and Coelho M.A.Z. (2009). Study of saline wastewater influence on activated sludge flocs through automated image analysis. *Journal of Chemical Technology & Biotechnology,* 84(4), 554-560.

Moon B.H., Seo G.T., Lee T.S., Kim S.S. and Yoon C.H. (2003). Effects of salt concentration on floc characteristics and pollutants removal efficiencies in treatment of seafood wastewater by SBR. *Water Science and Technology,* 47(1), 65-70.

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

Mosquera-Corral A., Gonzalez F., Campos J.L. and Mendez R. (2005b). Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds. *Process Biochemistry,* 40(9), 3109-3118.

Moussavi G., Barikbin B. and Mahmoudi M. (2010). The removal of high concentrations of phenol from saline wastewater using aerobic granular SBR. *Chemical Engineering Journal,* 158(3), 498-504.

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

Nicolau A., Dias N., Mota M. and Lima N. (2001). Trends in the use of protozoa in the assessment of wastewater treatment. *Research in Microbiology,* 152(7), 621-630.

Rene E.R., Kim S.J. and Park H.S. (2008). Effect of COD/N ratio and salinity on the performance of sequencing batch reactors. *Bioresource Technology,* 99(4), 839-846.

Sánchez O., Aspe E., Marti M.C. and Roeckel M. (2004). The effect of sodium chloride on the two-step kinetics of the nitrifying process. *Water Environment Research,* 76(1), 73-80.

Schwarzenbeck N., Erley R., Mc Swain B.S., Wilderer P.A. and Irvine R.L. (2004). Treatment of malting wastewater in a granular sludge sequencing batch reactor (SBR). *Acta Hydrochimica et Hydrobiologica,* 32(1), 16-24.

Su K.Z. and Yu H.Q. (2005). Formation and characterization of aerobic granules in a sequencing batch reactor treating soybean-processing wastewater. *Environmental Science and Technology,* 39(8), 2818-2827.

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(1-2), 227-233.

Tijhuis L., van Loosdrecht M.C.M. and Heijnen J.J. (1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioengineering,* 44(5), 595-608.

Uygur A. and Kargi F. (2004). Salt inhibition on biological nutrient removal from saline wastewater in a sequencing batch reactor. *Enzyme and Microbial Technology,* 34(3-4), 313-318.

Uygur A. (2006). Specific nutrient removal rates in saline wastewater treatment using sequencing batch reactor. *Process Biochemistry,* 41(1), 61-66.

Vázquez-Padín J.R., Figueroa M., Campos J.L., Mosquera-Corral A. and Méndez R. (2010a). Nitrifying granular systems: A suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology,* 74(2), 178-186.

Vázquez-Padín J.R., Mosquera-Corral A., Campos J.L., Méndez R., Carrera J. and Pérez J. (2010b). Modelling aerobic granular SBR at variable COD/N ratios including accurate description of total solids concentration. *Biochemical Engineering Journal,* 49(2), 173-184.

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

Wang Q., Du G.C. and Chen J. (2004). Aerobic granular sludge cultivated under the selective pressure as a driving force. *Process Biochemistry,* 39(5), 557-563.

Wang S.G., Liu X.W., Gong W.X., Gao B.Y., Zhang D.H. and Yu H.Q. (2007). Aerobic granulation with brewery wastewater in a sequencing batch reactor. *Bioresource Technology,* 98(11), 2142-2147.

Weber S.D., Ludwig W., Schleifer K.H. and Fried J. (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Applied and Environmental Microbiology,* 73(19), 6233-6240.

Wilen B.M., Gapes D. and Keller J. (2004). Determination of external and internal mass transfer limitation in nitrifying microbial aggregates. *Biotechnology and Bioengineering,* 86(4), 445-457.

Yilmaz G., Lemaire R., Keller J. and Yuan Z. (2008). Simultaneous nitrification, denitrification, and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. *Biotechnology and Bioengineering,* 100(3), 529-541.

Zhu S. and Chen S. (2002). The impact of temperature on nitrification rate in fixed film biofilters. *Aquacultural Engineering,* 26(4), 221-237.

Chapter 4:

Microbial community monitoring in an aerobic granular reactor treating fish canning effluents

Summary

Since the macro-scale aspects of the operation of an aerobic granular reactor for the treatment of fish canning effluents have been already studied (Chapter 3), it is of great interest to research the way the presence of salts (like NaCl) and the changes in the feeding composition affect the microbial populations of the granules. Therefore, in this work the rRNA approach (Amann *et al.*, 1995), as a cultivation-independent solution to the identification of microorganisms shaping the granules treating fish canning effluents, was used. A set of 16S rRNA gene sequences from the different bacterial groups forming the granules were retrieved by different molecular techniques (i.e. PCR-DGGE, cloning and sequencing). Furthermore the FISH technique was also used to gain deeper insight of the populations forming the granules which were found to be composed of ammonia-oxidizing and heterotrophic bacteria.

From the application of these molecular techniques a heterogeneous distribution of microbial populations inside the granules was observed, comprising members of the subclasses *Alpha-*, *Beta-* and *Gammaproteobacteria*. The predominance of members of the *Betaproteobacteria* was associated with the large genera of denitrifying and nitrifying bacteria present. Filamentous organisms belonging to genera *Chloroflexi* and *Thiotrix* were also identified. In the present case they did not provoke a detrimental effect on the structure of the granules, but they seemed to play an important role in their scaffolding.

The present study revealed that the heterotrophic bacterial community was composed of microorganism that were phylogenetically and metabolically diverse which ensured the stability of the ecosystem as a granule and sustained the performance of the reactor.

Keywords: Aerobic granules; fish canning effluents; microbial identification; microscopic techniques; molecular techniques; salt.

4.1. INTRODUCTION

The most effective way for removing organic compounds and nutrients from wastewater is the biological treatment. In conventional activated sludge systems, microorganisms assemble into small flocs (≤ 0.3 mm in size) but under special conditions, it is possible to promote the growth of bacteria in the form of biofilms or aerobic granules (around 2 mm in size) (Adav *et al.*, 2008).

The formation of aerobic granules requires specific conditions such as high hydrodynamic shear stress, feast-famine regime or a batch feeding strategy that provides strong selective pressures (Liu *et al.*, 2005). The effects of several key operating parameters on the reactor performance were comprehensively researched by the use of synthetic feeding (Liu and Tay, 2004).

Research efforts have also been dedicated to the study of aerobic granules for its application to the removal of organic matter, nitrogen and phosphorus compounds from industrial wastewater (Adav *et al.*, 2008; Campos *et al.*, 2009). So far, most of the research has been primarily focused on the macro-scale characterization of aerobic granular sludge systems but also their micro-scale structure has been deeply explored (Liu *et al.*, 2004; Ivanov *et al.*, 2005; Chen *et al.*, 2007; Zheng and Yu, 2007; Lemaire *et al.*, 2008b). Although most of them were performed on granular systems fed with synthetic wastewater containing a single carbon source.

However, detailed information on granule structure and nitrogen conversions in aerobic granules treating industrial wastewater is still absent (Lemaire *et al.*, 2008a). Thus the identification of the microbial communities involved in these systems is of interest. The complex nature of the wastewater, which contains diverse carbon sources along with a multitude of organic and inorganic compounds and some particulate matters, and the different substrate-degradation rates are likely to have an effect on the structure of aerobic granules and their ability to remove COD and/or nutrients. Moreover, the presence of chemicals or modifications in the feeding composition is expected to be responsible for changes in the microbial populations and might affect the structure and properties of aerobic granules.

Effluents from fish canning industries are a good example of complex wastewater that presents high variability in their composition depending on the season and type of manufactured product. The use of seawater in the process is common in these industries, with the consequent generation of wastewater containing large salt concentrations (Sánchez *et al.*, 2004). The afore mentioned advantages and versatility of the aerobic granular technology make these systems suitable to deal with this kind of wastewater with successful results (Figueroa *et al.*, 2008).

The efficiency and robustness of these biological treatments relies on the multiple microbial activities and interactions occurring in the aerobic granules. The introduction of molecular techniques based on ribosomal RNA made it possible to determine the composition and dynamics of microbial communities in biological systems and to identify the microbial key players for the different process types (Sanz and Köchling, 2007). The most commonly used molecular techniques for the identification of microorganisms in wastewater treatment systems are those based in the DNA amplification by means of polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE) or cloning. The differences in DGGE profiles were also used for describing ecological communities by using diversity indexes and species abundance patterns due to the likely influence of biodiversity on the function of ecosystems (Briones and Raskin, 2003). Finally, fluorescent *in situ* hybridization (FISH) technique is used for direct visualization and also quantification of the microorganisms based on phylogenetically different ribosomal RNA sequences using specific rRNA-targeted oligonucleotide probes.

Research developed to identify the main bacterial populations present in aerobic granules and to establish a rough correlation between the identified ones and the macroscopic operation of the reactor will provide relevant information for the scale up and application of this kind of systems.

4.2. OBJECTIVE

The aim of this study was to make micro-scale observations of the structure and microbial distribution of aerobic granules from a lab-scale SBR system fed with fish canning effluents containing high salt concentrations. The structure and microbial populations of the aerobic granules were examined using a wide range of molecular techniques including PCR, DGGE, cloning and FISH combined with confocal laser scanning microscopy (CLSM) using specific probes for the detected bacterial groups.

4.3. MATERIALS AND METHODS

4.3.1. Reactor description and operational conditions

The reactor description and the operational conditions of the SBR during the five operational stages which lasted 475 days were described in detail in Chapter 3.

4.3.2. Fluorescent *in situ* hybridization

Microbial populations were followed by the fluorescence *in situ* hybridization (FISH) technique. Granules from the reactor were collected, kept in their aggregated form or disaggregated, and fixed according to the procedure described by Amann *et al.*, (1995), with 4% paraformaldehyde solution. Entire granules were embedded in OCT reagent (Tissue-Tek; Miles, Ind.) prior to their cryosectioning. Slices with a thickness of 20 µm were cut at -19 °C,

and placed on the surface of poly-L-lysine coated microscopic slides. To achieve the granular biomass breakage, biomass was sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher). Hybridization was performed at 46 °C for 90 minutes, adjusting formamide concentrations at the percentages shown in Table 4.1. The used probes for *in situ* hybridization were 5' labeled with the fluorochromes FITC, Cy3 or Cy5. The samples were observed by confocal laser scanning microscopy (CLSM). Quantification of microbial populations, based on a biovolume fraction, was performed after the application of the FISH technique with the *Daime* software (Daims *et al.*, 2006).

Table 4.1. Probes used for FISH and the percentage of formamide (%F) used during hybridization.

Probe	Probe sequence (5'→3')	%F	Target organisms	Ref[a]
EUB338	GCTGCCTCCCGTAGGAGT	0-50	Bacteria domain	[1]
EUB338II	GCAGCCACCCGTAGGTGT	0-50	Planctomycetales	[2]
EUB338III	GCTGCCACCCGTAGGTGT	0-50	Verrucomicrobiales	[2]
ALF1B	CGTTCGYTCTGAGCCAG	20	Alphaproteobacteria	[3]
NIT3	CCTGTGCTCCATGCTCCG	40	*Nitrobacter* spp.	[4]
Competitor	CCTGTGCTCCAGGCTCCG			
PAR1244	GGATAACCCACTGTCACC	20	Paracoccus	[5]
BET42a	GCCTTCCCACTTCGTTT	35	Betaproteobacteria	[3]
Competitor	GCCTTCCCACATCGTTT			
Nso190	CGATCCCCTGCTTTTCTCC	55	Betaproteobacterial ammonia-oxidizing bacteria	[6]
NEU653	CCCCTCTGCTGCACTCTA	40	Most halophilic and halotolerant Nitrosomonas spp.	[7]
Competitor	TTCCATCCCCCTCTGCCG			
CTE	TTCCATCCCCCTCTGCCG	20	Comamonas sp., Acidovorax sp., Hydrogenophaga sp., Aquaspirillum sp.	[8]
MZ1	TCTGCCGTACTCTAGCCTT	45	Thauera spp. mzt1t	[9]
ZRA23	CTGCCGTACTCTAGTTAT	35	Most members of the Zoogloea lineage, not Z. resiniphila	[10]
GAM42a	GCCTTCCCACATCGTTT	35	Gammaproteobacteria	[3]
Competitor	GCCTTCCCACTTCGTTT			
G123T	CCTTCCGATCTCTATGCA	40	Thiothrix eikelboomii, T. nivea, T. unzii, T. fructosivorans, T. defluvii	[11]
Competitor	CCTTCCGATCTCTACGCA			
CFX1223	CCATTGTAGCGTGTGTGTMG	35	phylum Chloroflexi	[12]
GNSB941	AAACCACACGCTCCGCT	35	phylum Chloroflexi	[13]
CF319a	TGG TCC GTG TCT CAG TAC	35	most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*	[14]
CFB560	WCCCTTTAAACCCART	30	subgroup of *Bacteroidetes*	[15]
CFB562	TACGYWCCCTTTAAACCCA	30	subgroup of *Bacteroidetes*	[15]

[a][1] Amann *et al.*, 1990; [2] Daims *et al.*, 1999; [3] Manz *et al.*, 1992; [4] Wagner *et al.*, 1996; [5] Neef *et al.*, 1996; [6] Mobarry *et al.*, 1996; [7] Wagner *et al.*, 1995; [8] Schleifer *et al.*, 1992; [9] Lajoie *et al.*, 2000; [10] Rosselló-Mora *et al.*, 1995; [11] Kanagawa *et al.*, 2000; [12] Bjornsson *et al.*, 2002; [13] Gich *et al.*, 2001; [14] Manz *et al.*, 1996; [15] O'Sullivan *et al.*, 2002.

4.3.3. DNA extraction

Total community DNA was extracted from granules harvested at different operational days, following a bead beating protocol using a PowerSoil DNA soil extraction kit (MoBio Laboratories) following the manufacturer's instructions. The nomenclature of the samples and sampling days were as follows: sample 1: day 44; sample 2: day 79; sample 3: day 99; sample 4: day 143; sample 5: day 210; sample 6: day 268; sample 7: day 344; sample 8: day 368; sample 9: day 443; sample 10: day 470.

4.3.4. DGGE

Genomic DNA from all the samples was subjected to DGGE analysis. Briefly, the 16S rRNA gene hypervariable regions of bacteria V3-V5 were amplified using primers 341F-GC and 907R (Yu and Morrison, 2004). Primer 341F-GC included a GC-clamp at its 5' end (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'). In this case, PCRs were performed in a volume of 50 µL containing 1.25 U of Taq (TaKaRa ExTaq™ Hot Start Version; TaKaRa Bio Inc., Otsu, Siga, Japan), 1× ExTaq Buffer (2 mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 0.5 µM of primers and 100 ng of template DNA. After 9 min of initial denaturation at 95 °C, a touchdown thermal profile protocol was carried out, and the annealing temperature was decreased by 1 °C per cycle from 65 °C to 55 °C; followed by 20 additional cycles of 1 min of denaturation at 94 °C, 1 min of primer annealing at 55 °C, and 1.5 min of primer extension at 72 °C, followed by 10 min of final primer extension at 72 °C.

Approximately 800 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel with denaturing gradients that ranged from 40 to 75% (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1× TAE buffer using an INGENY PhorU system (Ingeny) at 100 V and 60 °C for 16 h. DGGE gels were stained with 1× TAE buffer containing SybrGold (Molecular Probes). Predominant DGGE bands were excised with a sterile razor blade, suspended in 50 µL of sterilized MilliQ water, stored at 4 °C overnight and reamplified by PCR using primers 341F-907R.

4.3.5. Statistical analysis of DGGE images

Digitized DGGE images were analyzed using the program Gel 2K (Svein Norland, Dept. of Biology, University of Bergen), which determines the presence or absence of bands (band patterns) based on grey level units and pixel intensity. The DGGE patterns were tested for similarity by using the Jaccard-Index that calculates the resemblance matrix. This matrix was used for the creation of a cladogram, which classifies together the closest obtained profiles.

4.3.6. 16S rRNA gene clone library

Almost complete 16S rRNA was amplified from genomic DNA using primers 616F and 630R as previously described (Juretschko *et al.*, 1998). PCR reaction using 100 ng of DNA from sample 8 was performed exactly as described above for DGGE.

16S rRNA amplicons were cloned and transformed into competent *E. coli* cells using a cloning kit according to the manufacturer's instructions (pGEM®-T Easy, Promega). Recombinant pGEM®-T Easy vectors were checked for their 16S rDNA amplicon inserts by PCR using primers M13F and M13R, which flank the pGEM®-T Easy cloning site. PCR products from recombinant clones were separated by electrophoresis in a 1% (w/v) agarose gel, stained with ethidium bromide and photographed under UV light using XLite system (Avegene).

4.3.7. Sequencing and phylogenetic analysis

In order to eliminate the excess of primers and dNTPs for sequencing reactions, the PCR products were digested at 37 °C for 1 hour using Shrimp Alkaline Phosphatase (SAP, 1 U/µL) and Exonuclease I (ExoI, 10 U/µL) (U.S.B. Corporation). The enzymes were afterwards inactivated by heating the samples at 80 °C for 15 min.

DNA extracted from DGGE bands was partially sequenced in both directions using primers F341, R907 (Edwards *et al.*, 1989) in the ABI PRISM™ sequence detection system. Those derived from clones with the proper insert size (1500 bp approx.) were sequenced in both directions using vector primers T7 and SP6. Sequences were inspected, assembled, subjected to the Check Chimera program of the Ribosomal Database Project (RDP) (Maidak *et al.*, 2000) and examined with the BLAST search alignment tool comparison software (BLASTN) (Altschul *et al.*, 1990) to detect the closest bacterial group to each strain among GenBank database.

Sequences were aligned with reference sequences obtained from GenBank using MAFFT (Katoh and Toh, 2008). The alignment obtained was edited using MacClade software (Maddison and Maddison, 2003) and transferred to jMODELTEST software version 3.6 (Posada and Crandall, 1998) as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment using the Akaike information criterion (AIC). Rate variation across sites, assuming a gamma distribution (0.4020) and a proportion of invariable sites (0.2270) were allowed. The best-fit models of nucleotide evolution for A, C, G and T were 0.2404, 0.2297, 0.3224 and 0.2076, respectively, with the rate matrix of the substitution model being 0.9002 (A/C), 1.8993 (A/G), 1.0584 (A/T), 0.7012 (C/G), 3.3843 (C/T) and 1.0000 (G/T). These calculated parameters were incorporated into the software PHYML (Guidon and Gascuel, 2003). Finally, the trees created by the PHYML were edited using the FIGTREE v1.1.2 software.

4.3.8. Nucleotide sequence accession numbers

The sequences obtained in this study through DGGE and clone sequencing have been deposited in the GenBank under accession numbers HQ184339 to HQ184402.

4.4. RESULTS AND DISCUSSION

4.4.1. Granule characteristics

Detailed description of the granules characteristics is presented in Chapter 3. Briefly, it was observed that during the start up of the SBR fluffy biomass structures were formed instead of the typical round shaped granules. These formed granules presented small fibrous structures in their surface that gradually disappeared and stable and mature granules were observed after day 75 of operation (S. II). Obtained granules presented very good physical properties; as an example, they exhibited density values around 60 g VSS/$L_{granule}$ and sludge volume index (SVI) around 30 mL/g TSS on day 208 of operation (S. III). The size of the granules gradually increased from 3 up to 6 mm until day 354 of operation (S. IV) when they broke up and the mean feret diameter decreased to 2.5 mm.

During stages S. III and S. IV (from days 149 to 375) the salinity of the influent decreased from 10.7 g NaCl/L down to 0.1 g NaCl/L. The main observed physical change of the biomass was the disappearance of filamentous overgrowth in the surface of the granules, probably due to the growth of protozoa and rotifers that graze on them.

4.4.2. Carbon and nitrogen removal

Experimental results obtained in the SBR reactor, in terms of nitrogen and organic matter removal, were described in Chapter 3. However, in order to permit a rapid visualization of the experimental data and the operational conditions corresponding to each biomass sampling day, the following figure and table are presented (Table 4.2 and Figure 4.1).

Table 4.2. Performance of the reactor in each biomass sampling day.

Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Day	**44**	**79**	**99**	**143**	**210**	**268**	**344**	**368**	**443**	**470**
Stage	**S. I**	**S. II**	**S. II**	**S. II**	**S. III**	**S. IV**	**S. IV**	**S. IV**	**S. V**	**S. V**
Salinity (g NaCl/L)	9	10.7	10.7	10.7	0.06	0.1	0.1	0.1	5.8	5.8
$OLR_{treated}$ (kg COD_s/m^3 d)	1.42	1.28	1.36	1.3	1.14	1.97	3.15	2.86	2.81	4.98
Rate (g COD_s/kg VSS d)	946	247	298	128	132	281	424	255	188	326
$NH_4^+{}_{inf}$ (mg N/L)	71	32	45	45	58	100	91	114	223	100
$NH_4^+{}_{eff}$ (mg N/L)	73	14	9	1	11	51	59	3	100	140
NO_x^- (mg N/L)	0	18	20	24	22	10	8	31	48	2
$NLR_{treated}$ (g N/m^3 d)	0	0	64	80	100	156	96	320	300	0
Rate (g N/kg VSS d)	0	0	14	7.7	11	22	13	28	2	0

It is remarkable that the organic removal efficiency was maintained over 85% during all the operational stages despite the variations of organic loading rate (OLR) from 1.72 up to 3 kg $COD_s/(m^3\ d)$. Stages S. I, S. II and S. III are characterized by a stable concentration of organic matter, around 150 mg TOC/L and also the development of nitritation after day 70 of operation. Subsequent increase of the treated loads and variations in the influent composition led to a high variability in terms of ammonia oxidation efficiencies during stages S. IV and S. V.

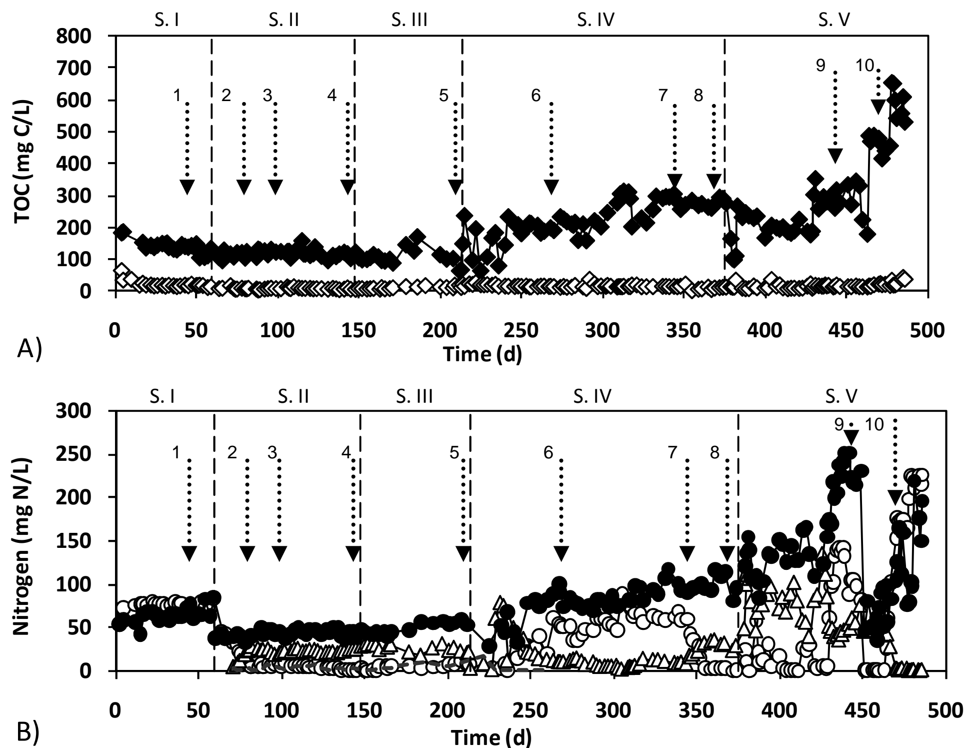


Figure 4.1. A) TOC concentration in the influent (◆) and in the effluent (◇). B) Ammonia concentration in the influent (●) and ammonia (○), nitrite (△) and nitrate (---) concentrations in the effluent. The arrows indicate the date of each of the collected samples.

4.4.3. DGGE and cloning analysis

Molecular DGGE profiles of PCR amplified bacterial rDNA 16S genes revealed the evolution of the initial microbial populations towards a different community (Figure 4.2). The DGGE profiles of the samples from the beginning of the operational period of the reactor (S. I and S. II) were close to each other (samples 1, 2, 3 and 4), whereas the profile of bacterial communities of the following stages were grouped together and clearly separated from the previous stages.

The transformation of activated sludge to form aerobic granules has been stated to be accompanied by changes in the microbial populations present. It has also been reported that similar time length is required to reach steady state conditions in the microbial communities composition than to reach steady state conditions in the reactor performance and physical characteristics of the formed granules (Liu *et al.*, 2010). In the present study, although the changes suffered during the biomass wash-out and the formation of the first granules are not reflected, a stable composition in the microbial populations present in the granules was observed during the transition from those elongated structures to granules (from day 40 to 75). Sample 4, corresponding to a stage with similar operational conditions than the previous ones, gave slightly different results. These results could indicate the evolution of the microbial populations towards a different community. Due to the operational changes (decrease of salinity and increase of loading rate) a shift on the bands was observed, and again evolved towards another microbial composition.

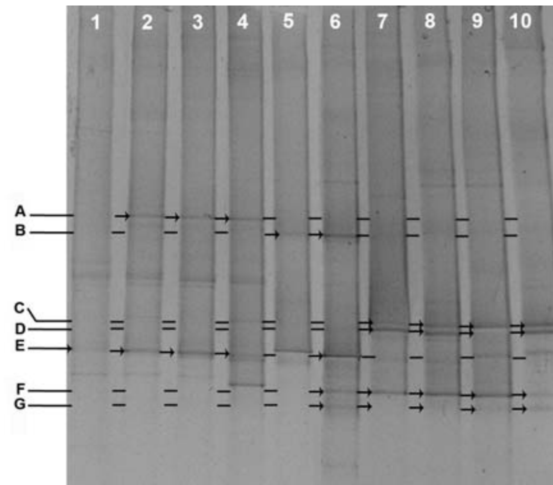


Figure 4.2. DGGE profiles of the bacterial communities in the reactor during the start up and operation of the aerobic granular reactor. The numbers on the top of the bands correspond with the following operational days, 1: day 44, 2: day 79, 3: day 99, 4: day 143, 5: day 210, 6: day 268, 7: day 344, 8: day 368, 9: day 443, 10: day 470.

Cluster analysis performed on the presence-absence of DGGE bands reflected these changes in the bacterial community and showed how they can be grouped according to the different operational stages, which correlates to the changes observed in the performance of the reactor (Figure 4.3). During the first two stages (S. I and S. II), when the operation of the reactor was more stable in terms of processes performance, the community composition was clearly different from that one at the end of the process (stages S. IV and S. V) with higher operational perturbations. Sample 5 (S. III) have no clear position and therefore could reflect the change in the salinity at that time of the operation. Although significant changes in the performance of the reactor were not observed at this point, the reduction of salinity

promoted changes in the outline of the granules (Figure 3.2, Chapter 3), and probably on the development of new microbial populations, those that were not able to develop under saline conditions (e.g. protozoa).

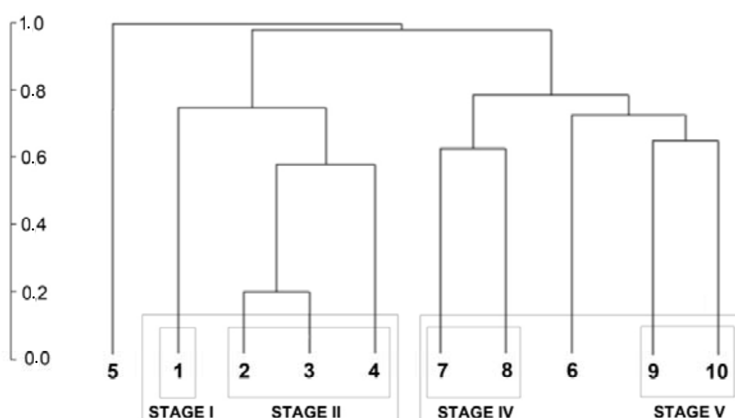


Figure 4.3. Cluster analysis from a similarity matrix generated from DGGE profiles according to the Jaccard similarity coefficient. The analysis showed a clear evolution of the bacterial community.

The different composition of sample 6 (S. IV) could reflect the perturbations caused in the system during the beginning of this stage and also the changes observed in the nitrogen removal during this stage. Samples 7 and 8 (S. IV) and samples 9 and 10 (S. V) presented differences in similarity, probably caused again by the salinity and the changing operational conditions suffered during stage S. V. However the change in the microbial community was not so sharp compared with the changes suffered during stage S. III. Although the non-adapted organisms could be inhibited and disappear from the system when the salinity was restored the system did not develop the same populations observed in stages S. I and S. II.

In order to identify the main microorganisms forming part of the microbial community, most intense bands were cut and sequenced and the results were aligned with previously published sequences from the NCBI database (Table 4.3).

Table 4.3. Affiliation of the bacterial 16S sequences retrieved from DGGE gel

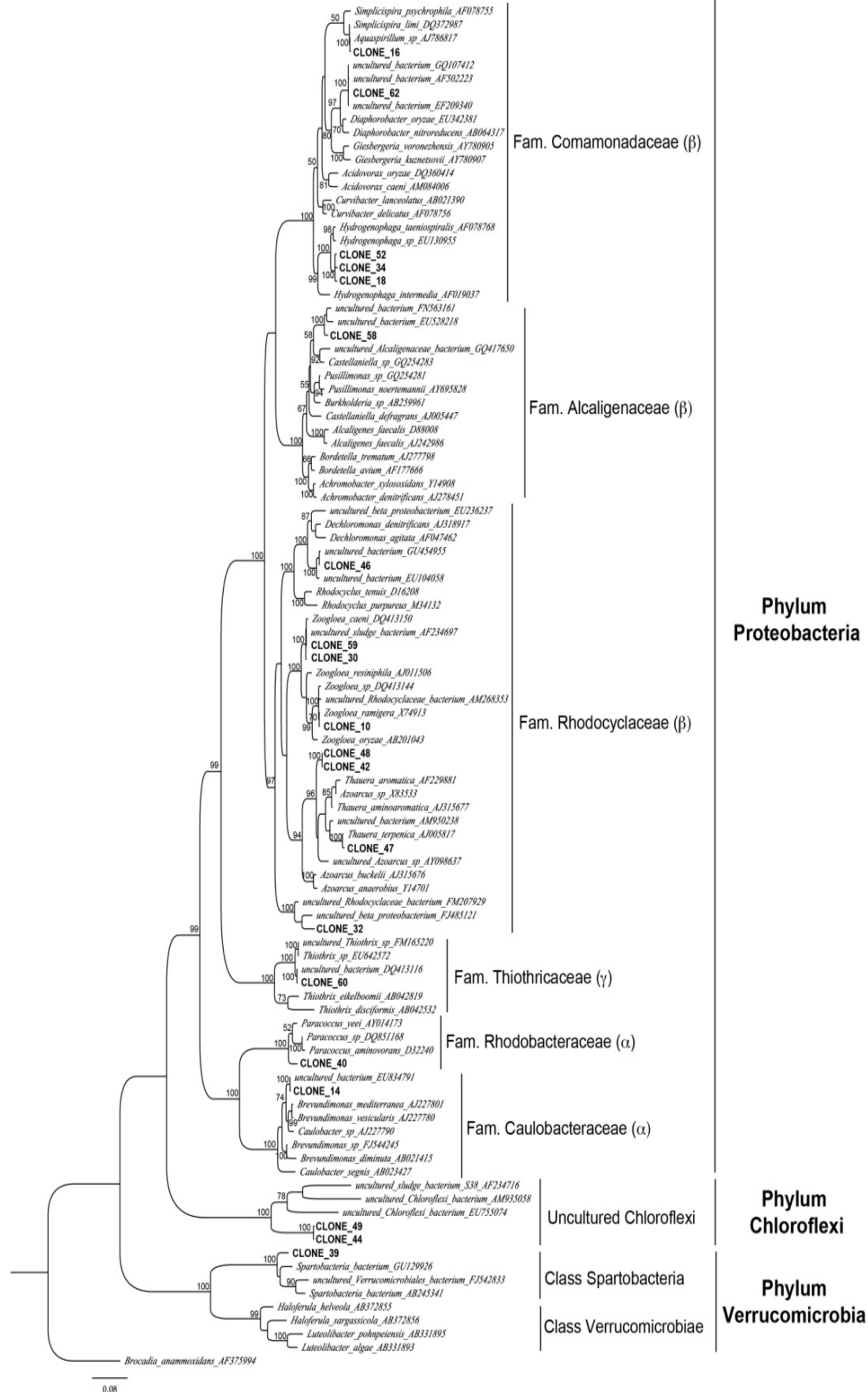
Band ID	Closest described	Identities (percent)	Phylum	Accession number
A	Uncultured Bacteroidetes bacterium clone BIhii16	526/528 (99%)	Bacteroidetes	AJ318142
B	Uncultured bacterium clone:12C-M51	526/527 (99%)	Bacteroidetes	AB205889
C/D	*Thiothrix caldifontana*	506/507 (99%)	Proteobacteria (Gamma)	EU642573
E	*Thauera* sp. R-26885	534/534 (100%)	Proteobacteria (Beta)	AM084104
F/G	Uncultured bacterium clone:NB-09	503/519 (96%)	Chloroflexi	AB117713

Bands belonging to the first two stages (S. I and S. II) were associated with *Bacteroidetes* phylum and *Thauera* genus while at the end of the operational period (S. IV and S. V) the community was characterized by the presence of *Thiothrix* and *Chloroflexi* in the granules and the disappearance of *Bacteroidetes*.

A clone library was done with the sample taken on day 368 (Sample 8) in order to identify more microorganisms from an operational day with mature granules performing organic matter and nitrogen removal processes. A number of 60 clones were obtained, but in the phylogenetic tree only those clones with 99% of sequence identity with type strains were represented (Figure 4.4). From the detected clones 17 belonged to the phylum *Proteobacteria* (Alpha-, Beta- and Gamma- subclasses) and 3 belonged to the phyla *Chloroflexi* and *Verrucomicrobia* (classes *Spartobacteria* and *Verrucomicrobiae*). From the clones belonging to *Betaproteobacteria*, 5 and 8 of them belonged to the families *Comamonadaceae* and *Rhodocyclaceae (*genera *Thauera* and *Zoogloea)*, respectively.

In several studies performed with sludge from urban wastewater treatment plants using molecular methods many heterotrophic microorganisms were reported to belong to these two families and they were attributed the role of performing the denitrification process (Rosselló-Mora *et al.*, 1995; Thomsen *et al.*, 2007) although it has been also observed that the community composition of the denitrifying bacteria is affected by the used carbon sources (Morgan-Sagastume et al., 2008). In several municipal activated sludge plants operated for carbon removal, nitrification and denitrification, *Betaproteobacteria* belonging to the genera *Thauera* and *Azoarcus* constituted from 3% to 16% of the total bacteria (Thomsen et al., 2007). *Azoarcus* together with *Zoogloea* and *Thauera* are abundant microorganisms in activated sludge and are believed to be the dominant denitrifiers also in some industrial treatment plants (Juretschko et al., 2002). Furthermore members of the families *Comamonadaceae* and *Rhodocyclaceae* (including *Thauera, Azoarcus* or *Zoogloea*) were previously found in aerobic granules by means of clone libraries (Li *et al.*, 2008; Adav *et al.*, 2010; De Sanctis *et al.*, 2010; Liu *et al.*, 2010).

Zoogloea spp. possesses also the ability of producing exopolysaccharides (EPS) and forming flocs (Farrah and Unz, 1976). *Zoogloea*-related bacteria have often been described as forming colonies in typical branched gelatinous matrices, the so-called *Zoogloea* fingers. Abundant *Zoogloea* sp. population was identified throughout the granulation process in aerobic granular reactors fed both with glucose or acetate, and kept their dominance under high organic loading conditions (OLR up to 19.5 kg COD/(m^3 d) (Li *et al.*, 2008; Adav *et al.*, 2009). Therefore, it was stated that these bacterial species likely played an important role in the formation and stabilization of the granule structure.


Simplicispira_psychrophila_AF078755
Simplicispira_limi_DQ372987
Aquaspirillum_sp_AJ786817
CLONE_16
uncultured_bacterium_GQ107412
uncultured_bacterium_AF502223
CLONE_62
uncultured_bacterium_EF209340
Diaphorobacter_oryzae_EU342381
Diaphorobacter_nitroreducens_AB064317
Giesbergeria_voronezhensis_AY780905
Giesbergeria_kuznetsovii_AY780907
Acidovorax_oryzae_DQ360414
Acidovorax_caeni_AM084006
Curvibacter_lanceolatus_AB021390
Curvibacter_delicatus_AF078756
Hydrogenophaga_taeniospiralis_AF078768
Hydrogenophaga_sp_EU130955
CLONE_52
CLONE_34
CLONE_18
Hydrogenophaga_intermedia_AF019037
Fam. Comamonadaceae (β)
uncultured_bacterium_FN563161
uncultured_bacterium_EU528218
CLONE_58
uncultured_Alcaligenaceae_bacterium_GQ417650
Castellaniella_sp_GQ254283
Pusillimonas_sp_GQ254281
Pusillimonas_noertemannii_AY695828
Burkholderia_sp_AB259961
Castellaniella_defragrans_AJ005447
Alcaligenes_faecalis_D88008
Alcaligenes_faecalis_AJ242986
Bordetella_trematum_AJ277798
Bordetella_avium_AF177666
Achromobacter_xylosoxidans_Y14908
Achromobacter_denitrificans_AJ278451
Fam. Alcaligenaceae (β)
uncultured_beta_proteobacterium_EU236237
Dechloromonas_denitrificans_AJ318917
Dechloromonas_agitata_AF047462
uncultured_bacterium_GU454955
CLONE_46
uncultured_bacterium_EU104058
Rhodocyclus_tenuis_D16208
Rhodocyclus_purpureus_M34132
Zoogloea_caeni_DQ413150
uncultured_sludge_bacterium_AF234697
CLONE_59
CLONE_30
Zoogloea_resiniphila_AJ011506
Zoogloea_sp_DQ413144
uncultured_Rhodocyclaceae_bacterium_AM268353
Zoogloea_ramigera_X74913
CLONE_10
Zoogloea_oryzae_AB201043
CLONE_48
CLONE_42
Thauera_aromatica_AF229881
Azoarcus_sp_X83533
Thauera_aminoaromatica_AJ315677
uncultured_bacterium_AM950238
Thauera_terpenica_AJ005817
CLONE_47
uncultured_Azoarcus_sp_AY098637
Azoarcus_buckelii_AJ315676
Azoarcus_anaerobius_Y14701
uncultured_Rhodocyclaceae_bacterium_FM207929
uncultured_beta_proteobacterium_FJ485121
CLONE_32
Fam. Rhodocyclaceae (β)
uncultured_Thiothrix_sp_FM165220
Thiothrix_sp_EU642572
uncultured_bacterium_DQ413116
CLONE_60
Thiothrix_eikelboomii_AB042819
Thiothrix_disciformis_AB042532
Fam. Thiothricaceae (γ)
Paracoccus_yeei_AY014173
Paracoccus_sp_DQ851168
Paracoccus_aminovorans_D32240
CLONE_40
Fam. Rhodobacteraceae (α)
uncultured_bacterium_EU834791
CLONE_14
Brevundimonas_mediterranea_AJ227801
Brevundimonas_vesicularis_AJ227780
Caulobacter_sp_AJ227790
Brevundimonas_sp_FJ544245
Brevundimonas_diminuta_AB021415
Caulobacter_segnis_AB023427
Fam. Caulobacteraceae (α)
Phylum Proteobacteria
uncultured_sludge_bacterium_S38_AF234716
uncultured_Chloroflexi_bacterium_AM935058
uncultured_Chloroflexi_bacterium_EU755074
CLONE_49
CLONE_44
Uncultured Chloroflexi
Phylum Chloroflexi
CLONE_39
Spartobacteria_bacterium_GU129926
uncultured_Verrucomicrobiales_bacterium_FJ542833
Spartobacteria_bacterium_AB245341
Class Spartobacteria
Haloferula_helveola_AB372855
Haloferula_sargassicola_AB372856
Luteolibacter_pohnpeiensis_AB331895
Luteolibacter_algae_AB331893
Class Verrucomicrobiae
Phylum Verrucomicrobia
Brocadia_anammoxidans_AF375994
0.08

In the previous page: **Figure 4.4.** Maximum likelihood phylogenetic tree of the clone sequences related to different bacterial groups. Clone sequences derived from this study are in bold while type sequences (and its accession numbers) for each clade are in regular characters. (α, β and γ indicate Alpha-,Beta- and Gamma- proteobacteria, respectively) Major groups including sequences derived from this study are named to the right of vertical lines. Phylogenies are based on alignments of 1326 bp. Bootstrap values of ≥ 50 percent are shown at branch nodes (1000 iterations). *Brocadia anammoxidans* was used as the outgroup.

Both DGGE and clone sequences revealed the presence of *Chloroflexi* and *Thiothrix* bacteria. Studies based on molecular detection showed the high diversity of *Chloroflexi* phylotypes either in natural or engineered environments (sediments, soils, lakes, microbial fuel cell systems and so on) (Yamada and Sekiguchi, 2009). Among engineered system, wastewater treatment plants are recognized as habitats with a high presence of members belonging to *Chloroflexi* e.g. membrane bioreactor (Miura *et al.*, 2007) or rotating disks (Kindaichi *et al.*, 2004). *Thiothrix* is a filamentous sulfur bacteria belonging to the class *Gammaproteobacteria* that was found to cause settleability problems in activated sludge processes and loss of solids from secondary clarifiers (Williams and Unz, 1985). The overgrowth of these bacteria also caused bulking and settling problems in aerobic granules cultivated with brewery wastewater (Weber *et al.*, 2007). Filamentous members of the phylum *Chloroflexi* have occasionally been associated with bulking incidences. In general, no industrial waste particularly favored the presence of filamentous *Chloroflexi*; they were detected in different types of industries as well as in municipal wastewater treatment plants (Bjornsson *et al.*, 2002; Kragelund *et al.*, 2007). Occurrence of filamentous growth has been previously reported in aerobic granular sludge SBR treating different kinds of wastewaters without causing operational problems, therefore it was stated that low-levels and moderate-levels of filamentous growth do not cause operational problems and may even stabilize the granule structure (Liu and Liu, 2006). In the present study both populations appeared in the case of mature granules while they were absent in the inoculated biomass.

4.4.4. FISH analyses

Identification: sample 8

Different FISH probes have been applied to disrupted aerobic granules sampled on day 368 (sample 8) according to the bacterial families detected after sequencing and following a hierarchical approach for the application of the probes.

From the application of probes ALF1b, BET42a and GAM42a only a small portion of the biomass was observed to hybridize with ALF1b probe indicating that they belonged to the *Alphaproteobacteria* (around 3%). A high percentage of bacteria belonged to the class *Betaproteobacteria* (around 45%), and also the presence filamentous bacteria was detected which belonged to *Gammaproteobacteria*.

One sequence from the clone library matched with *Paracoccus* genus, within *Alphaproteobacteria*. The use of the probe PAR1244 showed that almost all positive signals of this probe corresponded with those obtained after the application of probe ALF1b. Probes NIT3 and NTSPA712 for the identification of nitrite oxidizing bacteria *Nitrobacter* and *Nitrospira* were also tested, but negative results were obtained. This is in correspondence with the macroscopic results obtained in the reactor, since nitrate concentrations in the effluent were below 1 mg NO_3^--N/L.

A large number of sequences related to *Rhodocyclaceae* and *Comamonadaceae* families were retrieved from sequencing. Since in many cases they are identified as denitrifying bacteria and the performance of the reactor indicated the existence of denitrification, their abundance was checked. In order to distinguish the different genera of *Rhodocyclaceae*, probes MZ1 (*Thauera*) and ZRA23 (*Zoogloea*) were applied, and it was observed that their relative abundance was around 4% and 10%, respectively (Figure 4.5A). Bacteria belonging to the family *Comamonadaceae* was observed by the application of probe CTE and accounted for around 13% of the total population present in the sample (Figure 4.5B).

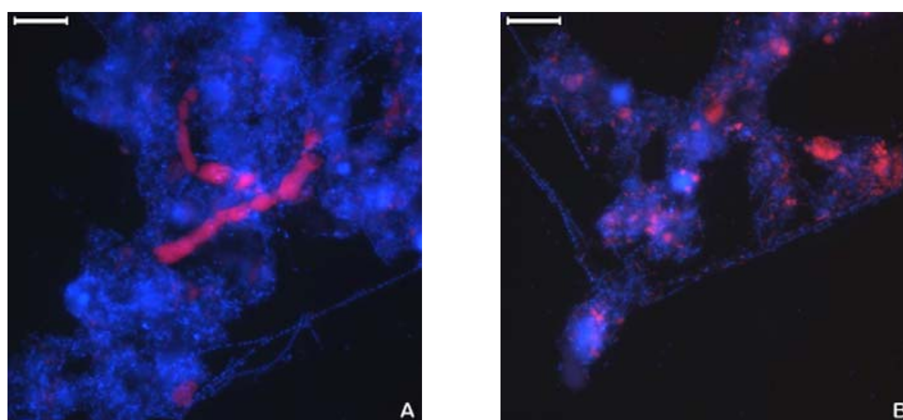


Figure 4.5. Day 368. *Zoogloea* bacteria in the biomass (fingers) (overlay of ZRA23-Cy3 and DAPI). *Comamonadaceae* bacteria in the biomass (overlay of CTE-Cy3 and DAPI). The bar indicates 25 µm.

The application of probe G123T corroborated that the most abundant filamentous bacteria present in the sample (*Gammaproteobacteria*) belonged to genus *Thiothrix*, and that their abundance was clearly higher than the one observed for *Chloroflexi* detected with the probe CFX1223 (Figure 4.6A,B). Obtained results indicated that the length of *Chloroflexi* bacteria was around 30 µm, much shorter if it is compared with the observed length of *Thiothrix* filamentous bacteria which ranged from 75 to 300 µm.

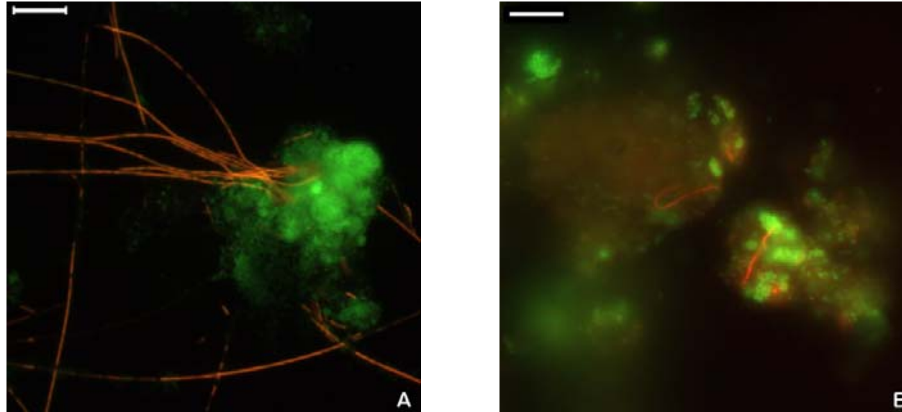


Figure 4.6. Day 368. A) Filaments of *Thiothrix* bacteria in the biomass (overlay of G123T-Cy3 and EUBmix-FITC); the bar indicates 25 µm. B) *Chloroflexi* bacteria (overlay of GNSB941-Cy3 and EUBmix-FITC); the bar indicates 10 µm.

From DGGE analysis the presence of members of the phylum *Bacteroidetes* in the samples from days 79 to 268 was determined. This is a broad phylum, formerly known as *Cytophaga-Flavobacter-Bacteroides* group that has been updated on the basis of 16S rRNA sequences in the last years and divided into four main classes: *Bacteroidia*, *Cytophagia*, *Flavobacteria* and *Sphingobacteria* (Bernardet and Holmes, 2010).

Bacteria belonging to the phylum *Bacteroidetes* are known to play an important role in the turnover of organic matter (Cottrell and Kirchman, 2000). It was reported that members of this phylum are heterotrophic gram negative bacteria, with various morphologies: from small rods to long filaments, and a mixture of physiological types: from obligate aerobes to obligate anaerobes. *Bacteroidetes* have been identified in wastewater treatment plants, a diverse range of freshwater (rivers and lakes) and marine ecosystems (O'Sullivan *et al.*, 2006; Kragelund *et al.*, 2008). FISH probes designed for this phylum (CF319a, CFB560 and CFB562) were applied.

When FISH was applied, it was observed that bacteria identified with probe CFB562 showed mostly rod-shaped bacteria (Figure 4.7A), whereas bacteria hybridized with probe CF319a showed mostly coccus-shaped bacteria (Figure 4.7B), and that CFB560 covered both. The percentage of positive hybridizations of probe CFB560 compared to DAPI was around 10%. The morphological differences could be related to the different target organisms of the probes within *Bacteroidetes*. Probe CFB560 was designed to target most of *Bacteroidetes*, CFB562 to target *Bacteroidetes* but excluding *Cytophaga* group (O'Sullivan *et al.*, 2002) whereas probe CF319a was designed to target within *Cytophaga* and *Flavobacter* (Manz *et al.*, 1996).

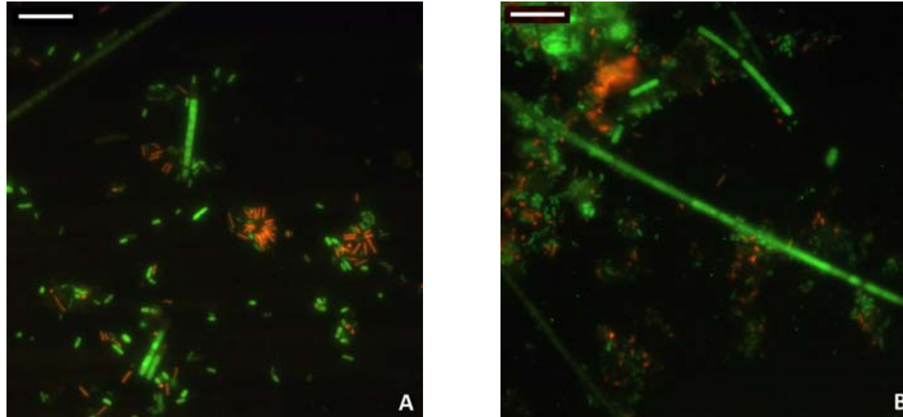


Figure 4.7. Day 368. A) Rod-shaped *Bacteroidetes* in the biomass (overlay of CFB562-Cy3 and EUB_{mix}-FITC). B) Coccus-shaped *Bacteroidetes* in the biomass (overlay of CF319a-Cy3 and EUB_{mix}-FITC). The bar indicates 10 µm.

The theoretical target range of probes designed to identify members of phylum *Bacteroidetes* was calculated using the sequences included in the RDP database (Ribosomal Database Project, release 10). Although the definition of the probes CFB560 and CFB562 is the same, in Table 4.4 the differences among the targeted number and the targeted classes can be observed. This result could explain the reason why the morphology of the bacteria observed by FISH with probes CFB562 and CF319a is so different.

Table 4.4. Theoretical target ranges of various CFB probes.

	No. of sequences with zero mismatches/no. tested (probe specificity)		
CFB group	**CFB560**	**CFB562**	**CF319a**
Phylum Bacteroidetes	589/603	279/603	357/603
Class Bacteroidia	118/121	95/121	22/121
Class Flavobacteria	278/280	2/280	269/280
Class Sphingobacterium	190/199	180/199	65/199
Class Cytophagia	57/60	55/60	6/60

Target range was calculated as the proportion of isolated type strains in the RDP small-subunit prokaryotic data set (release 10) which contained the probe target sequence with zero mismatches.

Although sequences related to ammonia oxidizing bacteria (AOB) were not obtained from DGGE or cloning, the FISH probes specific for these organisms were tested, since the performance of the reactor indicated the existence of ammonia oxidation to nitrite. Specific probes NSO190 and NEU653 for AOB were applied with fitting positives results for *Nitrosomonas*. From the results of NEU653 probe it was estimated that AOB accounted around 8% of the population (Figure 4.8). In spite of working with a disrupted sample, it was still possible to see the dense microbial aggregates characteristic of AOB.

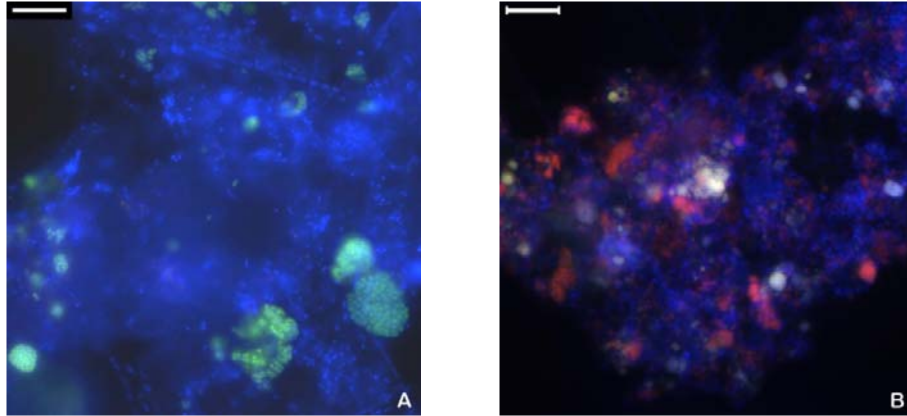


Figure 4.8. Day 368. A) *Nitrosomonas* clusters (overlay of NEU653-FITC and DAPI); the bar represents 10 µm. B) Relative abundance of *Nitrosomonas* (white) and *Betaproteobacteria* (overlay of NEU653-FITC, BET42a-Cy3 and DAPI); the bar represents 25 µm.

The application of the FISH technique to disrupted granules combined with the previous knowledge gained with sequencing allowed an easy and rapid identification and estimation of the abundance of the main bacterial groups that were present in the granules, however, a high percentage of microorganisms remained still unidentified (Figure 4.9).

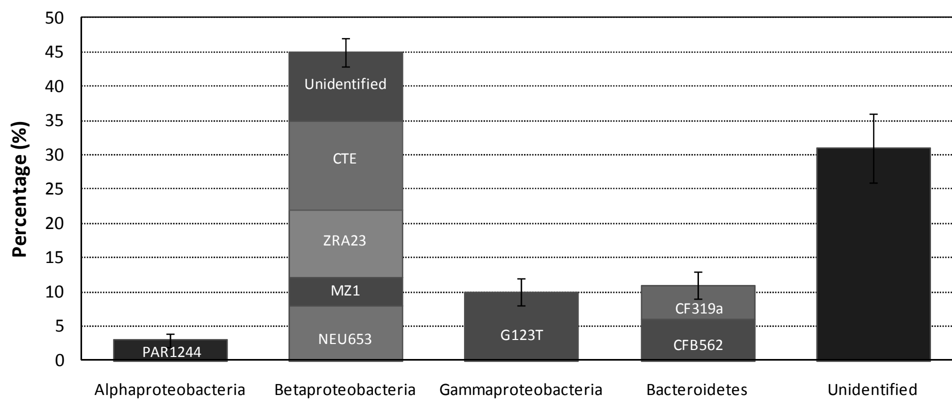


Figure 4.9. Estimation of the percentages of main microbial populations identified in the reactor on day 368 of operation.

The following step was to apply the probes that showed the most abundant microbial populations to sliced granules since it is known that aerobic granules may comprise several microbial layers composed of distinct bacterial species with various functional tasks, such as organic matter oxidation, nitrification or denitrification (Tay *et al.*, 2002; Aoi *et al.*, 2004; Weber *et al.*, 2007).

In the present case to maintain the integrity of the structure of the criosectioned granules in the slide during the different steps of the FISH procedure was difficult in spite of the use of an agarose cover layer. However to distinguish a fringe zone (with a depth of 300 µm) mainly composed of *Thiothrix* bacteria was possible. Clusters of AOB were also observed in this external layer (Figure 4.10) in coexistence with members of the genus *Zoogloea* and *Comamonadaceae* as indicated by the use of probes CTE and ZRA23.

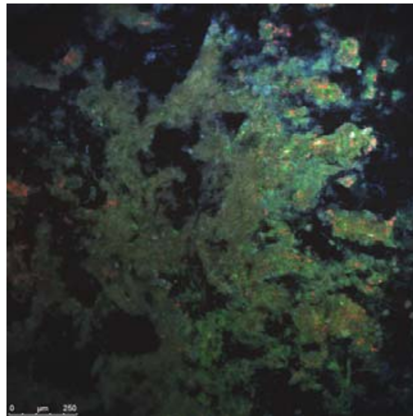


Figure 4.10. *Nitrosomonas* clusters in the external layer of the granule appear in red (NEU653-Cy3, EUBmix-FITC and DAPI).

The large microbial diversity found in aerobic granules has also led researchers to hypothesize that granulation is not only a function of specific microbiological groups but also is mainly related to the change of sludge morphology and substrate degradation. Therefore, the exact mechanisms involved in the successive stages of the aerobic granulation process have not yet been fully explained.

Evolution of certain bacterial population

Ammonia oxidizing bacteria:

The evolution along the time of *Nitrosomonas* population was followed also by the FISH technique (Figure 4.11). The percentage of abundance of these populations remained similar in comparison to the rest of the biomass during the time, in values around 8%. The main differences of the abundance percentages were found between the sample taken on day 58 and the rest. The former corresponds to the period when the nitrification process was firstly detected in the reactor so it is expected that this date corresponded to the sample containing the lowest amount of *Nitrosomonas* bacteria. Although a correlation between the amount of AOB detected by FISH and the nitrifying activity of a nitrifying trickling filter was previously reported (Biesterfeld *et al.*, 2003) in the present study, due to the low percentage of AOB, it was difficult to distinguish changes in the abundance during the different operational periods.

Moreover, the development of the fast growing heterotrophs due to the increased organic loading rate hindered the growth of AOB.

Nitrosomonas have no obligate salt requirement, but have a striking tolerance for increasing salt concentration up to 400 mM NaCl (25 g NaCl/L). Therefore, the presence of these microorganisms is not surprising in the aerobic granular reactor, with salt concentration up to 10 g NaCl/L in stages S. I and S. II. As it was previously reported in Chapter 3, in Stages III and IV the salt content in the feeding sharply diminished due to a change in the operational conditions of the fish canning industry. Due to their facultative halophylic character these changes were not reflected neither in the population dynamics nor in the performance of the *Nitrosomonas* population in the reactor.

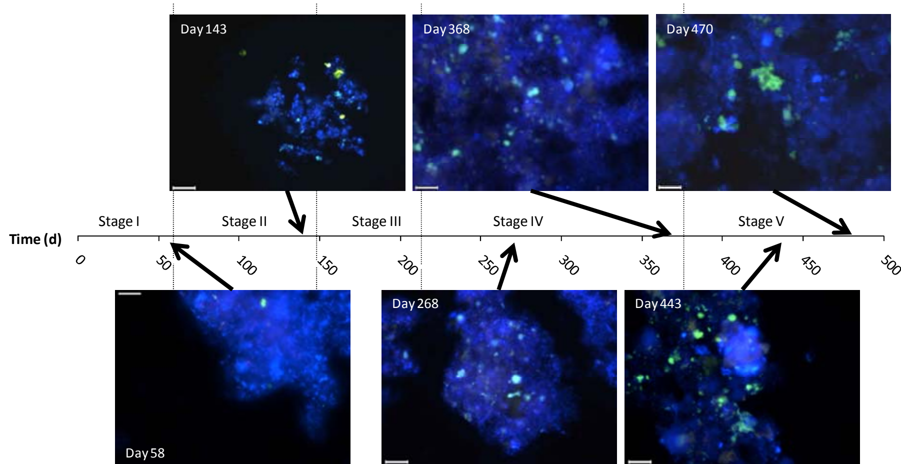


Figure 4.11. Evolution of *Nitrosomonas* population during the different operational stages by the use of the probe NEU653-FITC and DAPI. The bar indicates 25 µm except in sample from day 58 that indicates 10 µm.

The population of AOB was maintained during the whole operation of the reactor, however, the results corresponding to NOB were totally different. Only a small percentage of NOB was detected in the system with probe Nit3 (below 1%), in samples that corresponded to operating days from 79 to 210, when nitrate was measured in the effluent. These observations correlate with previous knowledge of the performance of heterotrophic granular reactors, that tends to limit the growth of NOB due to mass transfer limitations and the fast growth of heterotrophs (Vázquez-Padín *et al.*, 2010). An inhibitory effect was also observed due to free ammonia presence in the bulk liquid (Chapter 3).

Bacteroidetes:

The FISH probes designed for this phylum of Bacteroidetes (CF319a, CFB560 and CFB562) were applied on the samples taken on day 99 (sample 3) and on day 368 (sample 8).

A comparison between the results obtained from both samples revealed that on day 368 the amount of positive results given with probe CFB562 was higher than on day 99, as it was shown on Figure 4.12. Interestingly, many of the rod-shaped organisms detected with probe CFB562 were attached to the *Thiothrix* filaments.

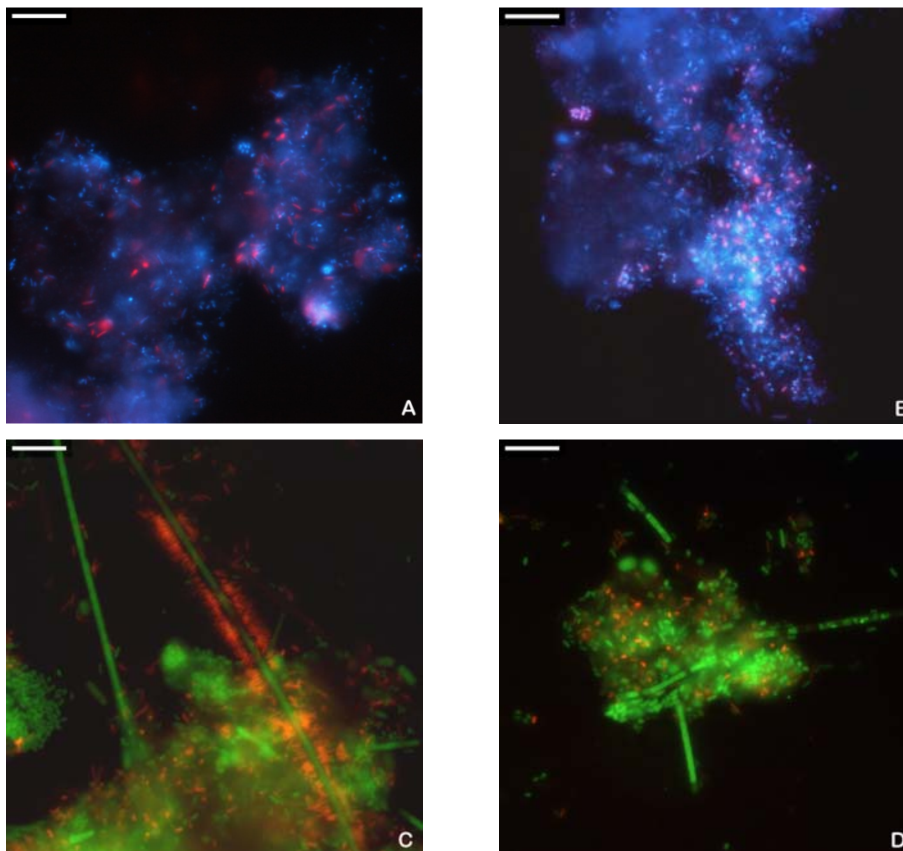


Figure 4.12. Day 99. A) Rod-shaped *Bacteroidetes* in the biomass (overlay of CFB562-Cy3 and DAPI). B) Coccus-shaped *Bacteroidetes* in the biomass (overlay of CF319a-Cy3 and DAPI). Day 368 C) Rod-shaped *Bacteroidetes* in the biomass (overlay of CFB562-Cy3 and EUB338$_{mix}$-FITC). D) Coccus-shaped *Bacteroidetes* in the biomass (overlay of CF319a-Cy3 and EUB338$_{mix}$-FITC).The bar indicates 10 µm.

Although the band corresponding to *Bacteroidetes* disappeared in the DGGE gel after day 268 (Figure 4.2) the detection of members belonging to this phylum by FISH on day 368

could be related to a non-selective binding of FISH probe. In this sense, this is the first report in which CFB562 probe was put into practice to analyze a biomass sample *in situ*. But it has to be also considered that lack of agreement between quantitative probing (e.g. FISH or quantitative PCR) and the clones abundance in the clone libraries has been previously reported (Juretschko *et al.*, 2002) since the PCR and sequencing are subject to a number of inherent biases and artefacts. So it is also possible that the organisms detected by FISH on day 368 were not represented in the sequenced clones due to an artifact caused by PCR or their random selection for sequencing, and that their DNA were represented in another DGGE band that was not selected for sequencing.

In any case, further research in order to clarify the presence of members of phylum *Bacteroidetes* is needed.

4.5. CONCLUSIONS

The use of molecular techniques such as DGGE and cloning enabled the identification of bacterial population forming part of the aerobic granules. Cluster analysis performed on the presence-absence of DGGE bands showed that changes in the bacterial community can be grouped according to the different operational stages, which correlates with the changes observed in the performance of the reactor. In the initial operational stages, selected bands indicated the presence of *Bacteroidetes* and *Thauera* whereas at the end *Thiothrix* and *Chloroflexi* were identified.

Different members of the subclasses *Alpha-*, *Beta-* and *Gammaproteobacteria* were identified by DGGE and cloning, and by the use of specific FISH probes. The abundance and evolution of nitrifying bacteria *Nitrosomonas* was visualized during the operation of the reactor. Distribution of denitrifying bacteria (*Zoogloea*, *Comamonadaceae* and *Thauera*) together with their spatial distribution in sliced granules was observed in the sample from day 368. On this day up to 60% of the total bacterial populations could be characterized.

Two different morphologies of *Bacteroidetes* bacteria were observed by the use of specific FISH probes, rod-shaped *Bacteroidetes* were targeted with CFB562 and coccus-shaped were targeted with CF319a.

Thiotrix and *Chloroflexi* were also present forming part of the structure of the granules, however, the presence of these filamentous bacteria did not compromise the integrity and the settling properties of the granules.

Although the composition of the culture media in which aerobic granules were developed also influences the microbial diversity, in this case the observed microorganisms were similar to those observed in conventional activated sludge systems operated in wastewater treatment plants.

4.6. REFERENCES

Adav S.S., Lee D.J., Show K.Y. and Tay J.H. (2008). Aerobic granular sludge: Recent advances. *Biotechnology Advances,* 26(5), 411-423.

Adav S.S., Lee D.J. and Lai J.Y. (2009). Functional consortium from aerobic granules under high organic loading rates. *Bioresource Technology,* 100(14), 3465-3470.

Adav S.S., Lee D.J. and Lai J.Y. (2010). Microbial community of acetate utilizing denitrifiers in aerobic granules. *Applied Microbiology and Biotechnology,* 85(3), 753-762.

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* 215(3), 403-410.

Amann R., Binder B.J., Olson R.J., Chisholm S.W., Devereux R. and Stahl D.A. (1990). Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial-populations. *Applied and Environmental Microbiology,* 56(6), 1919-1925.

Amann R., Ludwig W. and Schleifer K.H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews,* 59(1), 143-169.

Aoi Y., Tsuneda S. and Hirata A. (2004). Transition of bacterial spatial organization in a biofilm monitored by FISH and subsequent image analysis. *Water Science and Technology,* 49(11-12), 365-370.

Bernardet J.-F. and Holmes B. (2010). International Committee on Systematics of ProkaryotesSubcommittee on the taxonomy of Flavobacterium and Cytophaga-like bacteria. *International Journal of Systematic and Evolutionary Microbiology,* 60(3), 712-714.

Biesterfeld S., Russell P. and Figueroa L. (2003). Linking nitrifying biofilm structure and function through fluorescent in situ hybridization and evaluation of nitrification capacity. *Water Environment Research,* 75(3), 205-215.

Bjornsson L., Hugenholtz P., Tyson G.W. and Blackall L.L. (2002). Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal 9. *Microbiology,* 148(8), 2309-2318.

Briones A. and Raskin L. (2003). Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Current Opinion in Biotechnology,* 14(3), 270-276.

Campos J.L., Figueroa M., Mosquera-Corral A. and Mendez R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering,* 1, 136-151.

Cottrell M.T. and Kirchman D.L. (2000). Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Applied and Environmental Microbiology,* 66(12), 5116-5122.

Chen M.Y., Lee D.J. and Tay J.H. (2007). Distribution of extracellular polymeric substances in aerobic granules. *Applied Microbiology and Biotechnology,* 73(6), 1463-1469.

Daims H., Bruhl 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(3), 434-444.

Daims H., Lucker S. and Wagner M. (2006). Daime, a novel image analysis program for microbial ecology and biofilm research. *Environmental Microbiology,* 8(2), 200-213.

De Sanctis M., Di Iaconi C., Lopez A. and Rossetti S. (2010). Granular biomass structure and population dynamics in Sequencing Batch Biofilter Granular Reactor (SBBGR). *Bioresource Technology,* 101(7), 2152-2158.

Edwards U., Rogell T., Blöker H., Emde M. and Böttger E.C. (1989). Isolation and direct complete nucleotide determination of entire genes-characterization of a gene coding for 16S-ribosomal RNA. *Nucleic Acids Research,* 17, 7843-7853.

Farrah S.R. and Unz R.F. (1976). Isolation of exocellular polymer from Zoogloea strains Mp6 and 106 and from activated-sludge. *Applied and Environmental Microbiology,* 32(1), 33-37.

Figueroa M., Mosquera-Corral A., Campos J.L. and Mendez R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. *Water Science and Technology,* 58(2), 479-485.

Gich F., Garcia-Gil J. and Overmann J. (2001). Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. *Archives of Microbiology,* 177(1), 1-10.

Guidon S. and Gascuel O. (2003). PHYML-A single, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood *Systematic Biology,* 52(5), 696-704.

Ivanov V., Tay S.T.L., Liu Q.S., Wang X.H., Wang Z.W. and Tay J.H. (2005). Formation and structure of granulated microbial aggregates used in aerobic wastewater treatment. *Water Science and Technology,* 52(7), 13-19.

Juretschko S., Timmermann G., Schmid M., Schleifer K.H., Pommerening-Roser A., Koops H.P. and Wagner M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. *Applied and Environmental Microbiology,* 64(8), 3042-3051.

Juretschko S., Loy A., Lehner A. and Wagner M. (2002). The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Systematic and Applied Microbiology,* 25(1), 84-99.

Kanagawa T., Kamagata Y., Aruga S., Kohno T., Horn M. and Wagner M. (2000). Phylogenetic analysis of and oligonucleotide probe development for Eikelboom Type 021N filamentous bacteria isolated from bulking activated sludge. *Applied and Environmental Microbiology,* 66(11), 5043-5052.

Katoh K. and Toh H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics,* 9(4), 286-298.

Kindaichi T., Ito T. and Okabe S. (2004). Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. *Applied and Environmental Microbiology,* 70(3), 1641-1650.

Kragelund C., Levantesi C., Borger A., Thelen K., Eikelboom D., Tandoi V., Kong Y., Waarde J.v.d., Krooneman J., Rossetti S., Thomsen T.R. and Nielsen P.H. (2007). Identity, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. *FEMS Microbiology Ecology,* 59(3), 671-682.

Kragelund C., Levantesi C., Borger A., Thelen K., Eikelboom D., Tandoi V., Kong Y., Krooneman J., Larsen P., Thomsen T.R. and Nielsen P.H. (2008). Identity, abundance and ecophysiology of filamentous bacteria belonging to the Bacteroidetes present in activated sludge plants. *Microbiology,* 154(3), 886-894.

Lajoie C.A., Layton A.C., Gregory I.R., Sayler G.S., Taylor D.E. and Meyers A.J. (2000). Zoogleal clusters and sludge dewatering potential in an industrial activated-sludge wastewater treatment plant. *Water Environment Research,* 72, 56-64.

Lemaire R., Webb R.I. and Yuan Z. (2008a). Micro-scale observations of the structure of aerobic microbial granules used for the treatment of nutrient-rich industrial wastewater. *The ISME Journal,* 2(5), 528-541.

Lemaire R., Yuan Z., Blackall L.L. and Crocetti G.R. (2008b). Microbial distribution of Accumulibacter spp. and Competibacter spp. in aerobic granules from a lab-scale biological nutrient removal system. *Environmental Microbiology,* 10(2), 354-363.

Li A.j., Yang S.F., Li X.y. and Gu J.d. (2008). Microbial population dynamics during aerobic sludge granulation at different organic loading rates. *Water Research,* 42(13), 3552-3560.

Liu Y. and Tay J.H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances,* 22(7), 533-563.

Liu Y., Wang Z.W., Qin L., Liu Y.Q. and Tay J.H. (2005). Selection pressure-driven aerobic granulation in a sequencing batch reactor. *Applied Microbiology and Biotechnology,* 67(1), 26-32.

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., 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(2), 143-148.

Liu Y.Q., Kong Y.H., Zhang R., Zhang X., Wong F.S., Tay J.H., Zhu J.R., Jiang W.J. and Liu W.T. (2010). Microbial population dynamics of granular aerobic sequencing batch reactors during start-up and steady state periods. *Water Science and Technology,* 62(6), 1281-1287.

Maddison D.R. and Maddison W.P. (2003) MacClade 4: Analysis of phylogeny and character evolution.Version 4.06, Sinauer Associates, Sunderland, Massachusetts.

Maidak B.L., Cole J.R., Lilburn T.G., Parker C.T., Saxman P.R., Stredwick J.M., Garrity G.M., Li B., Olsen G.J., Pramanik S., Schmidt T.M. and Tiedje J.M. (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Research,* 28(1), 173-174.

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(4), 593-600.

Manz W., Amann R., Ludwig W., Vancanneyt M. and Schleifer K.-H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology,* 142(5), 1097-1106.

Miura Y., Watanabe Y. and Okabe S. (2007). Significance of *Chloroflexi* in performance of submerged membrane bioreactors (MBR) treating municipal wastewater. *Environmental Science & Technology,* 41(22), 7787-7794.

Mobarry B.K., Wagner M., Urbain V., Rittmann B.E. and Stahl D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology,* 62(6), 2156-2162.

Morgan-Sagastume F., Nielsen J.L. and Nielsen P.H. (2008). Substrate-dependent denitrification of abundant probe-defined denitrifying bacteria in activated sludge. *Fems Microbiology Ecology,* 66(2), 447-461.

Neef A., Zaglauer A., Meier H., Amann R., Lemmer H. and Schleifer K.H. (1996). Population analysis in a denitrifying sand filter: conventional and in situ identification of Paracoccus spp. in methanol-fed biofilms. *Applied and Environmental Microbiology,* 62(12), 4329-4339.

O'Sullivan L.A., Weightman A.J. and Fry J.C. (2002). New Degenerate Cytophaga-Flexibacter-Bacteroides-Specific 16S Ribosomal DNA-Targeted Oligonucleotide Probes Reveal High Bacterial Diversity in River Taff Epilithon. *Applied and Environmental Microbiology,* 68(1), 201-210.

O'Sullivan L.A., Rinna J., Humphreys G., Weightman A.J. and Fry J.C. (2006). Culturable phylogenetic diversity of the phylum 'Bacteroidetes' from river epilithon and coastal water and description of novel members of the family Flavobacteriaceae: Epilithonimonas tenax gen. nov., sp. nov. and Persicivirga xylanidelens gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* 56(1), 169-180.

Posada D. and Crandall K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics,* 14(9), 817-818.

Rosselló-Mora R.A., Wagner M., Amann R. and Schleifer K.H. (1995). The abundance of *Zoogloea ramigera* in sewage treatment plants. *Applied and Environmental Microbiology,* 61(2), 702-707.

Sánchez O., Aspe E., Marti M.C. and Roeckel M. (2004). The effect of sodium chloride on the two-step kinetics of the nitrifying process. *Water Environment Research,* 76(1), 73-80.

Sanz J.L. and Köchling T. (2007). Molecular biology techniques used in wastewater treatment: An overview. *Process Biochemistry,* 42(2), 119-133.

Schleifer K.H., Amann R., Ludwig W., Rothermund C., Springer N., Dorn S., Galli E., Silver S. and Witholt B. (1992) Pseudomans: Molecular biology and biotechnology, pp. 127-135, American Society for microbiology.

Tay J.H., Ivanov V., Pan S. and Tay S.T.L. (2002). Specific layers in aerobically grown microbial granules. *Letters in Applied Microbiology,* 34(4), 254-257.

Thomsen T.R., Kong Y. and Nielsen P.H. (2007). Ecophysiology of abundant denitrifying bacteria in activated sludge. *FEMS Microbiology Ecology,* 60(3), 370-382.

Vázquez-Padín J.R., Figueroa M., Campos J.L., Mosquera-Corral A. and Méndez R. (2010). Nitrifying granular systems: A suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology,* 74(2), 178-186.

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(2), 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(1-2), 237-244.

Weber S.D., Ludwig W., Schleifer K.H. and Fried J. (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Applied and Environmental Microbiology,* 73(19), 6233-6240.

Williams T.M. and Unz R.F. (1985). Filamentous sulfur bacteria of activated sludge: characterization of Thiothrix, Beggiatoa, and Eikelboom type 021N strains. *Applied and Environmental Microbiology,* 49(4), 887-898.

Yamada T. and Sekiguchi Y. (2009). Cultivation of Uncultured Chloroflexi Subphyla: Significance and Ecophysiology of Formerly Uncultured Chloroflexi 'Subphylum I' with Natural and Biotechnological Relevance. *Microbes and Environments,* 24(3), 205-216.

Yu Z.T. and Morrison M. (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* 70(8), 4800-4806.

Zheng Y.M. and Yu H.Q. (2007). Determination of the pore size distribution and porosity of aerobic granules using size-exclusion chromatography. *Water Research,* 41(1), 39-46.

Chapter 5:

Treatment of high loaded swine slurry in an aerobic granular reactor [1,2]

Summary

An aerobic granular sequencing batch reactor was evaluated as an alternative to the anaerobic digestion for organic matter removal from swine slurry. Furthermore this system provided with simultaneous nitrogen is removal. The aerobic granular sludge was obtained in the system after few days from the start-up. On day 140 of operation, the biomass properties were: 5 mm of average diameter, SVI of 32 mL/g TSS and density around 55 g $VSS/L_{granule}$. Organic matter removal efficiencies up to 87% and nitrogen removal efficiencies up to 70% were achieved when organic and nitrogen loading rates (OLR and NLR) of 4.4 kg COD/(m^3 d) and 0.81 kg N/(m^3 d) were applied. However, nitrogen removal processes were negatively affected when the OLR was rose up to 7.0 kg COD/(m^3 d) and the NLR was of 1.26 kg N/(m^3 d). In order to be able to treat the swine slurry without dilution the operational cycle of the reactor was modified by reducing the volumetric exchange ratio from 50 to 6%.

Keywords: Aerobic granular sludge; nitrogen; organic matter; SBR; swine slurry; solids.

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[1]Figueroa, M., Val del Río A., Campos J.L., Mosquera-Corral, A., and Mendez, R. (2011). Treatment of high loaded swine slurry in an aerobic granular reactor. *Water Science and Technology,* 63: 1808-1814.

[2] Val del Río A., Figueroa M., Arrojo B., Mosquera-Corral A., Campos J.L., García-Torriello G. and Méndez R. (2011). Aerobic granular SBR systems applied to the treatment of industrial effluents. *Journal of Environmental Management.* (accepted)

5.1. INTRODUCTION

In the last 50 years, intensive livestock production has increased significantly across Europe to produce cheap and balanced food supply. The livestock facilities have increased in size and are concentrated mainly in specific regions. This has resulted in large increases in the quantities of farm yard manure and slurry produced which, in many cases, are inadequately managed. Farm characteristics and animal densities vary widely across Europe with generally less intensive agriculture and thus lower density in the Southern European countries than in the Northern countries. In the case of swine production, for example, the density in Northern Europe is about ten times greater than that in the Southern countries (Figure 5.1.A). In Spain, the swine population is around 26×10^{6} farm animals and the estimated swine slurry production is around 48×10^{6} ton/year (FAOSTAT, 2009). The Spanish production is mainly located in Autonomous Communities such as Catalonia, Aragon and Castile and León where the 63% of the total pig population is located (Figure 5.1.B).

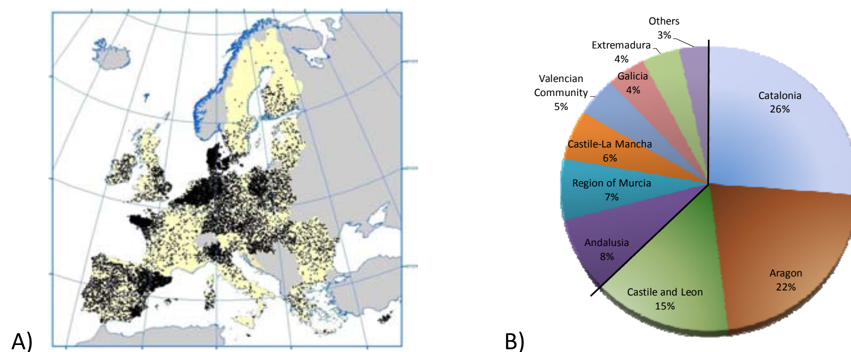


Figure 5.1. A) Number of sows by region. EUROSTAT (2008), 1 dot = 1000 sows. B) Distribution of stock heads in Spain in 2009

This intensive production is creating scenarios where the livestock waste is not being correctly disposed and valorised. In earlier practice, wastes generated in the farms were normally used as nutrients for agriculture, but the amount of produced manure that can be applied to the land has a limit that depends on the soil characteristics and the current nutrient levels. Manure (solid fraction with solids content above 15%) and pig slurry (solids content below 15%) contains the following four primary contaminants that impact the water quality: nitrogen (mainly ammonia), phosphorous, organic matter (complex carbohydrates) and pathogens which are believed to be responsible for severe water pollution, diseases, eutrophication and odour problems.

Therefore, the use of physical, chemical or biological processes is needed to avoid contamination of soil and groundwater from this wastewater. The objective is to treat and/or use the manure in an ecologically sustainable manner, reintegrating nutrients in the

environment without accumulating harmful substances above an acceptable level (Lampert, 2003).

A currently adopted swine wastewater treatment practice includes anaerobic digestion of carbonaceous compounds for waste treatment and energy recovery. The European Union energy policies are evolving towards the development of anaerobic digestion (European Parliament, 2008) with the aim of reducing emissions of volatile organic compounds, controlling odours, mineralising nutrients, improving its fertilising proprieties and energy recovering by biogas production. This process can be carried out in a reactor operated at mesophilic conditions but also in deep earthen outdoor basins (anaerobic lagoons). The drawback of this technology is that present organic nitrogenous compounds, such as proteins, aminoacids or urea, are mainly reduced to ammonia which is not further degraded in anaerobic conditions. The produced nitrogen rich effluent has to be treated before discharge, usually by means of nitrification and denitrification processes.

Anaerobic digesters of swine wastewater can treat organic loading rates up to 6 kg COD/(m^3 d) but are limited in terms of nitrogen removal (Deng *et al.*, 2006). The organic load treated in conventional aerobic systems designed for simultaneous organic matter and nitrogen removal is generally lower than 3 kg COD/(m^3 d) and 0.3 kg N/ (m^3 d), respectively although removal efficiencies up to 97% and 98%, respectively have been achieved (Magrí and Flotats, 2008). Most of the times the effluent generated from the anaerobic digesters present low COD/N ratios, meaning the amount of organic matter is then insufficient to achieve complete denitrification. Thus to overcome this drawback, different solutions regarding nitrogen removal from swine effluents based in the use of upflow multi-layer bioreactors, tubular biofilm photobioreactors or intermittent aeration systems are under study (Cheng and Liu, 2001; An *et al.*, 2007; de Godos *et al.*, 2009).

Aerobic sequencing batch reactors (SBR) are widely applied for wastewater treatment because their flexible operating strategy and compact construction (Mace and Mata-Alvarez, 2002). These systems have also been applied to the treatment of livestock waste (Su *et al.*, 1997; Obaja *et al.*, 2005; Beline *et al.*, 2008; Han *et al.*, 2008). When a SBR reactor is operated under aerobic conditions, if the accurate operational parameters that promote the development of aerobic granular biomass are applied, the benefits of this system are increased. These advantages include a decrease of the implantation surface requirements, high treated organic loads, etc. It is known that the granules are characterized by the presence of aerobic and anoxic layers which enable the possibility of carrying out simultaneously organic matter and nitrogen removal (de Kreuk *et al.*, 2005).

When a system consisting of an aerobic granular reactor is compared to another based on anaerobic digestion several questions must be taken into account. On one hand, the operational cost related to the aeration costs are expected to be higher than in the case of a system comprising an anaerobic reactor plus a second nitrification-denitrification system. On

the other hand, the use of a flexible system with a high H/D that allows a simultaneous organic matter and nitrogen removal treating high OLR could be an alternative for those farms with low slurry production and high space requirements.

5.2. OBJECTIVE

The objective of this work was to study the feasibility of the use of the aerobic granular technology to treat pig slurry effluents. The start up and the performance of the aerobic granular system will be evaluated in terms of carbon and nitrogen removal efficiencies and special attention will be paid to the physical properties of the granular biomass. Finally, the effect of the diminution of the operational volumetric exchange ratio was tested in order to be able to treat raw slurry.

5.3. MATERIALS AND METHODS

5.3.1. Analytical methods

Analytical determinations of total suspended solids (TSS), volatile suspended solids (VSS), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), sludge volume index (SVI), conductivity and pH was carried out according to the Standard Methods (APHA-AWWA-WPCF, 2005). Concentrations of total organic carbon (TOC) and inorganic carbon (IC) were measured with an analyser (TOC-5000, Shimadzu). Chemical Oxygen Demand (COD) soluble (COD_s) or total (COD_t) was determined by a semi-micro method (Soto *et al.*, 1989). Biomass density was determined using dextran blue (Beun *et al.*, 1999). The morphology and size distribution of the granules were measured regularly by using an image analysis procedure (Tijhuis *et al.*, 1994) with a stereomicroscope (Stemi 2000-C, Zeiss). NO_x concentration in the gas phase was determined by a 1 mL sample injected into the gas chromatograph (5890 Series II, HP).

5.3.2. Reactor description and operational conditions

A laboratory scale sequencing batch reactor with a working volume of 1.5 L and a height to diameter ratio of 5.5 was used. A condenser with cool water was installed on the top of the reactor in order to retain the possibly present aerosols leaving the reactor with the off-gas. A volume of 1 L of feeding was kept in a side-tank before its addition to the reactor, in order to avoid sharp changes of temperature at the beginning of each cycle due to the storage of the feeding at around 4 °C.

Air was supplied continuously during the aeration phase by a diaphragm pump (Laboport N86, KNF) through an air sparger located at the bottom of the reactor with an air flow of 3.5 L/min. The formation of small bubbles guaranteed the complete mixture of the liquid. The volumetric exchange ratio (VER) was firstly fixed at 50% (Figure 5.2.A) and then decreased down to 6% (Figure 5.2.B). A set of two peristaltic pumps was used to introduce the feeding solution (on top of the reactor) and to discharge the effluent. A programmable

logic controller (PLC) (S7-224CPU, Siemens) controlled the actuations of the pumps and valves, and regulated the different periods of the operational cycle.

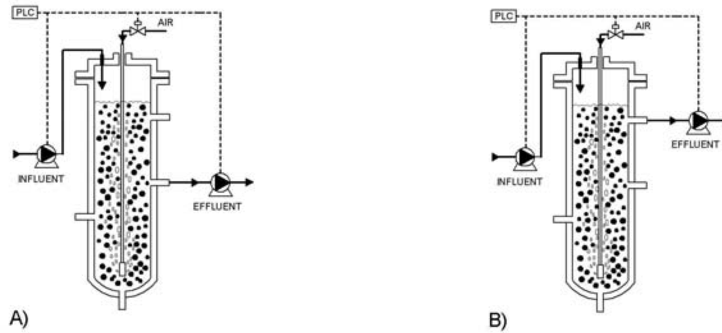


Figure 5.2. Experimental set-up of the granular SBR. Setup A) corresponds with a volumetric exchange ratio (VER) of 50% and B) with a VER of 6%.

A volume of 0.5 L of flocculent activated sludge, collected from the nitrifying-denitrifying system in operation in a municipal WWTP (Calo, Milladoiro), was used as inoculum. This sludge presented a Sludge Volume Index (SVI) of 115 mL/g TSS and a biomass concentration of 2.1 g VSS/L.

The reactor was operated in a sequencing batch mode, in cycles of 3 h distributed as follows: 171 minutes of aeration, 1 minute of settling and 5 minutes of effluent withdrawal and 3 minutes of feeding.

The slurry fed to the reactor was obtained from a pig farm located in San Marcos (Santiago de Compostela). The wastewater was sieved through 1 mm diameter mesh and settled overnight. The effluent was kept refrigerated at 4 °C to avoid degradation processes before its supply to the reactor.

The reactor operation was divided in two different stages according to the conditions shown in Table 5.1.

- During the first stage (Stage I) the slurry was diluted with tap water to achieve the appropriate organic and nitrogen loading rates (OLR, NLR) for the start-up. Along this period, both applied organic and nitrogen loads were stepwise increased by changing the dilution ratio of the raw slurry.

- During Stage II raw slurry was supplied to the SBR without dilution. OLR and NLR were kept in similar values to those used during Stage ID. For this purpose, the volumetric exchange ratio was reduced from 50% to 6% and therefore, the hydraulic retention time varied from 0.25 to 1.88 d.

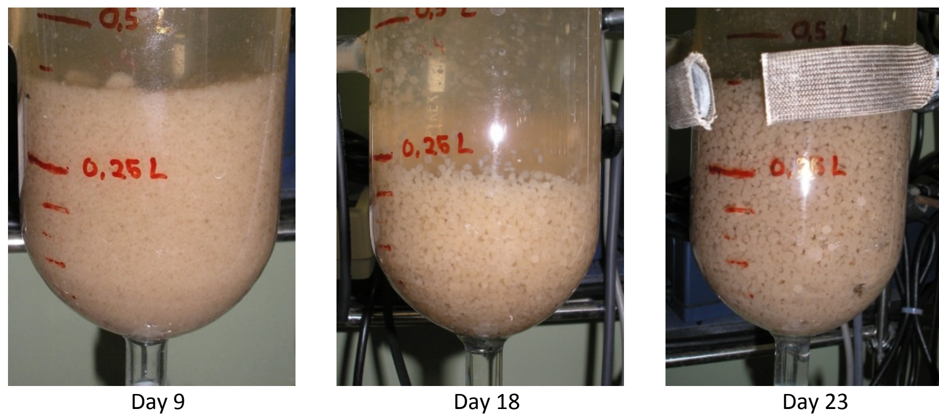
Table 5.1. Feeding composition and applied loading rates to the SBR along Stages I and II.

	Stage I				Stage II
	S. IA	S. IB	S. IC	S. ID	
Days	1-50	51-79	80-107	108-228	229-300
Dilution ratio	1:25	1:20	1:15	1:8	--
COD_s (mg/L)	560 ± 68	871 ± 73	1088 ± 111	1754 ± 280	13689 ± 1277
COD_t (mg/L)	579 ± 170	1038 ± 72	1430 ± 76	2093 ± 335	15932 ± 2627
OLR (kg COD_s/(m^3 d))	2.21 ± 0.26	3.50 ± 0.29	4.40 ± 0.45	7.0 ± 0.91	7.30 ± 0.68
NH_4^+-N (mg/L)	95 ± 8	149 ± 14	203 ± 23	313 ± 30	1823 ± 496
NLR (kg N/(m^3 d))	0.39 ± 0.03	0.60 ± 0.06	0.81 ± 0.09	1.26 ± 0.14	0.96 ± 0.27
pH	7.40 ± 0.20	7.64 ± 0.10	7.68 ± 0.10	7.10 ± 0.2	7.07 ± 0.10
COD/N ratio	5.79	5.89	5.45	5.59	7.60
Conductivity (µS/cm)	1600 ± 132	1939 ± 131	2637 ± 175	3276 ± 300	10403 ± 1372
VSS (g/L)	0.09 ± 0.03	0.11 ± 0.03	0.18 ± 0.02	0.23 ± 0.04	4.9 ± 1.8
TSS (g/L)	0.10 ± 0.05	0.11 ± 0.08	0.19 ± 0.03	0.24 ± 0.05	5.9 ± 1.5

5.4. RESULTS AND DISCUSSION

5.4.1. Granule formation and properties

The evolution of the granulation process during the first 23 days of operation (Stage I) can be observed in the images from Figure 5.3. A rapid increase in the feret diameter occurred from days 0 to 25 and it remained almost invariable until the increase of loading rate corresponding to Stage IB.



Day 9 Day 18 Day 23

Figure 5.3. Evolution of the granulation process during the first 23 days of operation (S. IA).

Only five days after the inoculation of the reactor the washout of most of the inoculated biomass and the appearance of small granules took place. After 10 days of operation the

average feret diameter of the granular biomass was of 1.87 mm (Figure 5.4A) and the volatile solids content inside the reactor was of 1.27 g VSS/L (Figure 5.4B) with a SVI value of 87 mL/g TSS (Figure 5.4C).

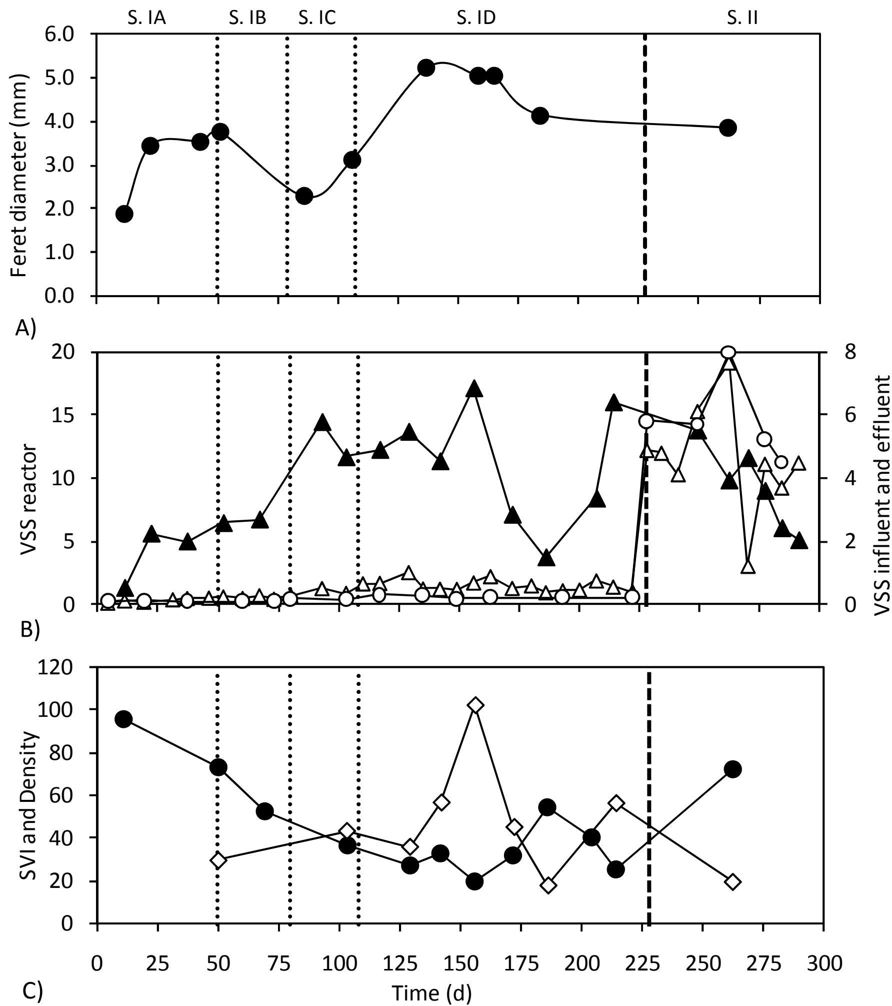


Figure 5.4. A) Mean feret diameter (mm) of the granules. B) Concentration of total suspended solids (g TSS/L) in the reactor (▲), volatile suspended solids (g VSS/L) in the influent (○) and in the effluent (△). C) SVI (mL/g TSS) (●) and density (g VSS/$L_{granule}$) (◇) of the granules.

A sharp increase of solids concentration up to 5.2 g VSS/L was produced until day 25 (S. IA) simultaneously to the development process of the aerobic granules. This value increased proportionally to the increase of fed OLR in each period (Table 5.1) until reaching a concentration of 16.1 g VSS/L on day 158 (S. ID) (Figure 5.4B). The calculated overall biomass yield during this period was of 0.19 g VSS/g COD. Until day 158, the mean feret diameter of

the granules varied between 2.0 and 4.0 mm, until reaching in this date a value of 5.2 mm (Figure 5.4A) while the SVI gradually decreased and reached a value of 20 mL/g TSS (Figure 5.4C). The outline of the granules during these stages was smooth (Figure 5.5A,B,C) but the structure was compact and the surface homogeneous. The density of the granules followed a similar pattern to the solids concentration inside of the reactor and increased simultaneously to the increase of OLR, and ranged from 25 to 50 g VSS/$L_{granule}$ from days 25 to 125. This parameter reached a maximum value of 100 g VSS/$L_{granule}$ on day 150 (Figure 5.4C).

The increase of biomass from days 130 to 158 (S. ID) caused that the granules reached the level of the discharge port and were partially washed-out with the effluent withdrawal. Therefore, the solids concentration inside the reactor decreased from day 158 on. Furthermore, at the same time, it was observed the breakage of some granules. The increase of both density and diameter of granules could cause limitations of internal and/or external oxygen transfer rate (Mosquera-Corral *et al.*, 2005; Lemaire *et al.*, 2008) and promoted the formation of loose zones (Figure 5.5D) that detached from the granule and that had different settling properties. From days 158 to 186 a sharp decrease of solids content inside of the reactor was observed that varied from 16.1 to 3.7 g VSS/L together with an increase of the SVI up to 54 mL/g TSS.

A similar event of granules washout and breakage previously occurred in the reactor treating wastewater from a fish canning industry (Chapter 3). In both cases, the concentration of biomass inside of the reactor was over 10 g VSS/L, with values of 16.1 g VSS/L and 11.0 g VSS/L for the reactor treating slurry and fish canning effluents, respectively, while the feret diameter of the granules reached values above of 5 mm.

In the periodical episodes (days 115-125 and 160-170) when the biomass volume inside the reactor reached the level of the discharge port the biomass concentration in the effluent was up to 1.1 g VSS/L. These fluctuations in the solids concentration in the effluent caused that the solids retention time (SRT) in the system diminished. During stages SI.A, SI.B and SI.C this value was maintained around 11 days, but with the further increase of solids in the effluent during stage SI.D this value diminished down to 5 days. In both cases, the values are still much lower than the 70 days found in aerobic granular sludge reactors fed with acetate as sole carbon source (de Kreuk et al., 2005).

At the beginning of Stage II, the biomass concentration in the reactor had recovered its previous value of 15 g VSS/L, although the overgrowth of filamentous structures in the granules surface was observed (Figure 5.5E,F). The SVI was of 32 ± 11 mL/g TSS and the density around 50 g VSS/$L_{granule}$. During this stage, the reduction of the VER from 50 to 6% significantly influenced the stability of the system. This parameter allowed the control of the maximum applicable loading rate but affected the minimal imposed settling velocity, which decreased from 8 to 2 m/h. Settling velocity is as a tool of selection for fast-settling granular sludge versus slow settling flocculent sludge, as proposed by Beun *et al.*, (2002). When this

value is too low, the amount of biomass washed out from the reactor per cycle decreases and thereby it accumulates inside the reactor meaning that granules and flocculent biomass coexist (Schwarzenbeck *et al.*, 2005; Wang *et al.*, 2006). In the case that flocculent biomass overgrows the granular one, the system could lost the removal efficiency and the biomass retention capacity.

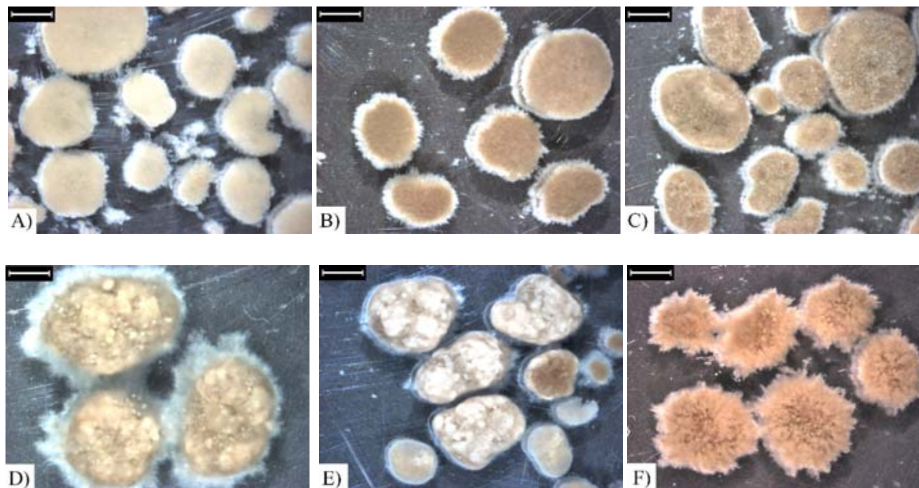


Figure 5.5. Stereomicroscope images of aerobic granules on days A) 22 (S. IA), B) 43 (S. IA), C) 106 (S. IB), D) 165 (S. ID), E) 184 (S. ID) and F) 262 (S. II). The bar represents 2 mm.

In the present study, the change in the VER promoted a quick growth of microorganisms in the form of flocculent biomass that was accumulated inside the system. This biomass was still able to settle during 1 minute, therefore, it was difficult to wash it out during the first seven days of operation of Stage II (Figure 5.6) due to the formation of a supernatant phase. During the remaining days two different phases were observed inside the reactor during settling. One was constituted by granules and the second by flocculent biomass.

The solids content in the influent, that in Stage II was around 6 g TSS/L (Table 5.1) influenced the accumulation and development of flocculent biomass in the system and also affected the solids concentration in the effluent, that was around 4 g TSS/L (Figure 5.4B). Moreover, it was observed the appearance of filamentous and finger type structures on the surface of the granules, as previously presented in Figure 5.5F. The growth of these filamentous structures could be also related to the solids concentration in the influent, since it was recently shown how the particulate and slowly biodegradable substrates, in the form of suspended solids and colloidal substrate, influenced the morphology of aerobic granules and the performance of aerobic granular reactors (de Kreuk *et al.*, 2010). In the cited study it was shown that particulate substances were consumed at certain regions of the granule surface instead of being accumulated inside the granules as occurred for soluble substrates, and the

occurrence of substrate gradients in the granule due to the local consumption of substrates stimulated the growth of these filaments structures.

In spite of the global increase of suspended solids inside the system, previously formed granules maintained its structure along the time. However, it was observed that the concentration of biomass in the form of granules inside the reactor decreased gradually because of the fact that sometimes they were trapped in these flocculent solids and were washed out from the reactor with them.

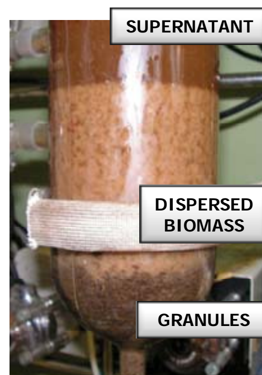


Figure 5.6. Settling phase during the cycle operation of the aerobic granular reactor on day 228. A supernatant phase, the flocculent biomass layer and the aerobic granules bed can be observed.

In order to avoid the predominance of flocculent solids inside the reactor when treating influent streams with high concentrations of solids several alternatives are possible: to add a previous step to reduce the solids concentration such as a solid-liquid separator or an anaerobic step to produce the hydrolysis and fermentation of substrates and also to reduce the settling time to increase the settling velocity to values over 8 m/h in order to wash out part of the non-settled biomass.

Regarding the physical properties of the biomass besides of the appearance of the granules and the mean feret diameter, it is also of great interest to know their distribution in terms of percentage in volume. This data provides information regarding the size of granules which contribute the most to the biomass content inside the system and indicate the degree of use of the biomass in the granules. The bigger the granules the larger the amount of biomass without activity in their volume.

The volumetric distribution corresponding to biomass samples collected in four different days is shown in Figure 5.7 as an example of the evolution of this parameter during the whole operational period. The increase in the mean feret diameter (Figure 5.4A) is also reflected in the volumetric distribution, with the displacement of the bars towards the right side of the histogram (higher mean feret diameter) (Figure 5.7).

As an example, it can be observed that in the first days of operation the volumetric size distribution is similar to a normal distribution curve (Figure 5.7A). On day 43 (Figure 5.7B), even though the mean feret diameter was 3.55 there were a high percentage of granules with the feret diameter between 3 and 7 mm, but also groups of granules with a diameter below to 3 mm that contributed in around 1% of the total volume each group. The wider volumetric distribution was observed on day 158, with the bars of the histogram distributed along all the formed groups (Figure 5.7C) indicating the presence of granules covering different sizes. On day 262 of operation the major contribution to the volumetric distribution was due to granules with a diameter that ranged from 3 to 7 mm (Figure 5.7D).

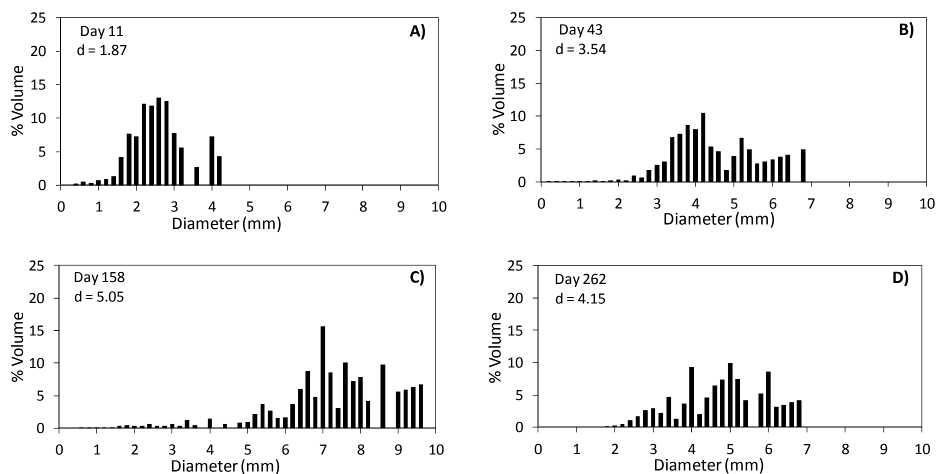


Figure 5.7. Volumetric size distribution (%). A) Day 11 (S. IA), B) day 43 (S. IA), c) day 158 (S. ID) and d) day 262 (S. II).

5.4.2. Organic matter removal

During the start up of the system, an OLR of 2.2 kg COD/(m^3 d) was applied to promote the formation of the granules (S. IA). Since the removal efficiency in terms of organic matter and the physical stability of the granules were maintained, the OLR was stepwise increased along Stage I up to an applied OLR of 7 kg COD/(m^3 d) (Table 5.1). The removal of the dilution water in the feeding and the decrease of VER during Stage II permitted to keep the OLR in a value around 7 kg COD/(m^3 d) (Figure 5.8).

In spite of these changes in the OLR, the system removal efficiency was maintained in similar values along the two different operational stages: 87±4% and 91±2%, Stages I and II respectively (Figure 5.8). The fraction of COD that remained in the effluent was around 10% of that contained in the feeding and corresponded to the slowly biodegradable or non biodegradable fraction that is present in swine slurry, as reported by Shin *et al.*, (2005).

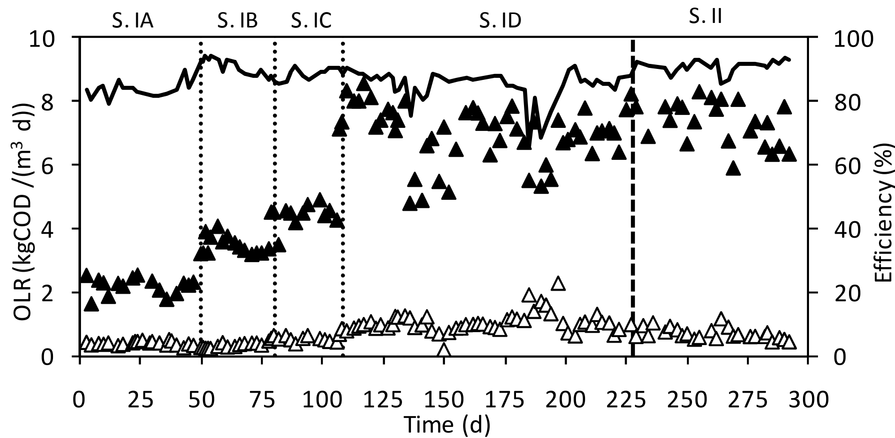


Figure 5.8. OLR (kg COD/(m^3 d)) in the influent (▲) and in the effluent (△) of the SBR, and COD removal efficiency (—) in stages S. I and S. II of operation.

The feeding pattern is considered as an important operational parameter that influences the aerobic granulation, therefore the maximum applied OLR value has to be carefully chosen. It has been found that the applied OLR can exert a microbial selection pressure on granulation process and affect the performance of granules by selection and enrichment of different bacterial species (Moy *et al.*, 2002). From previous studies it was stated that the increase of the OLR increases the possibility of aerobic granules to become unstable due to the outgrowth of filamentous microorganisms and the possible presence of anaerobic bacteria in the inner zone of big granules caused by oxygen limitations (Zheng *et al.*, 2006). However, the OLR threshold for the instability of aerobic granules heavily depends on the composition of the treated substrate and other operating parameters, including hydraulic shear force, settling time, volumetric exchange ratio, etc. For instance, acetate-fed aerobic granules could sustain a organic loading rate of 9 kg COD/(m^3 d) and disintegrate when treating 12 kg COD/(m^3 d) in the work developed by Chen *et al.*, (2008), however, with the same substrate, Moy *et al.*, (2002) found out that the granules disintegrated when the applied OLR reached a value of 9 kg COD/(m^3 d). In these cases the operational conditions, like different airflow rate, influenced the limit conditions of the system. In the present study, even though the granules suffered an episode of destabilization on day 158, after 50 days of operation under an OLR of 7.0 kg COD/(m^3 d) they could recover and keep on treating such an OLR during stage S. ID.

The presence of a feast and famine regime have been also reported as a factor that controls the granule structure during the start-up and operation of aerobic granular reactors (McSwain *et al.*, 2004). Therefore, the evolution of the organic matter concentration along one operational cycle was measured in different periods (Figure 5.9) to determine the length of the feast phase. The effect of the increase of OLR in each operational period was reflected

on the time needed to accomplish the biodegradable COD consumption and therefore the length of the feast and famine periods.

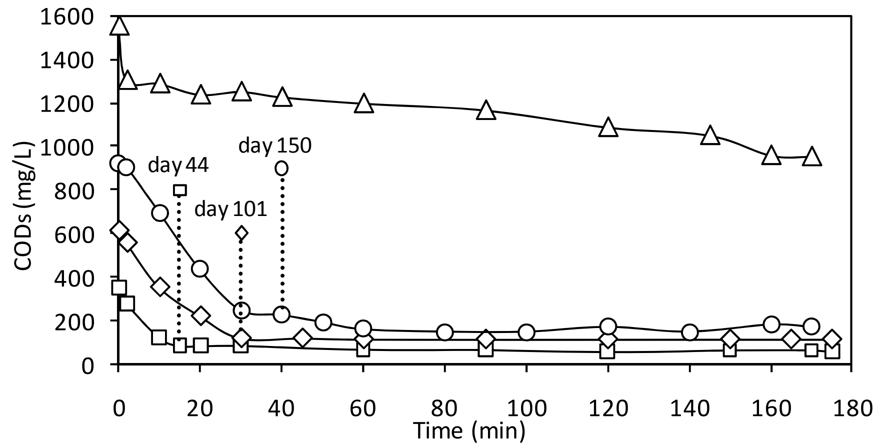


Figure 5.9. Evolution of CODs concentration (mg COD/L) along one operational cycle in days (□) 44 (OLR=2.2 kg COD/(m^3 d)), (◇) 101 (OLR=4.4 kg COD/(m^3 d)), (○) 150 (OLR=7.0 kg COD/(m^3 d)) and (△) 270 (OLR=7.2 kg COD/(m^3 d)). Each dotted line indicates the end of the feast phase according to the changes in the removed COD.

According to the obtained results, the feast phase varied from 15 min on day 44 (S. IA) to around 35 min on day 150 (S. ID). In the case of day 270 (S. V) a sharp decrease of the COD concentration in the first 5 min of the cycle was observed, however, this decreasing tendency continued smoothly during the rest of the cycle (Figure 5.9) and it was difficult to establish the length of the feast phase. In each of the analyzed cycles the correlation between the end of the COD removal and the increase of dissolved oxygen (DO) concentration was also observed. Therefore the latter was used to verify the length of the feast phase (Figure 5.10) and to determine it in the case of the cycle from day 270 which length of the feast phase was of 50 min.

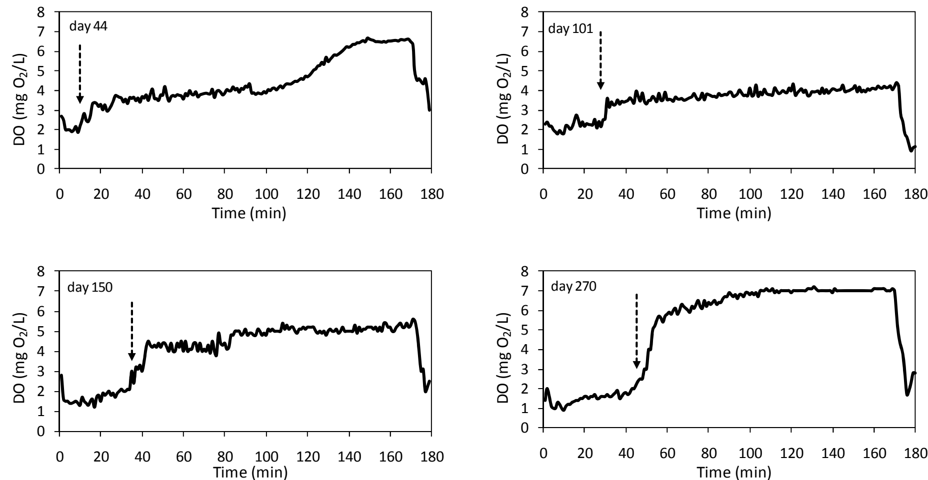


Figure 5.10. Dissolved oxygen concentration (mg O_2/L) in the bulk liquid during one cycle in the operational days 44 (S. IA), 101 (S. IC), 150 (S. ID) and 270 (S. II). Each dotted line represents the end of the feast phase according to the DO profile.

To keep the feast/famine ratio in low values is necessary to assure the integrity of the granule as it was stated by Liu and Tay (2007). These authors compared the performance of three different reactors operated with three feast/famine ratios and observed that a ratio of 0.88 min/min caused the instable physical integrity of the granules. Optimal performance in the system was achieved when this ratio was of 0.21 min/min.

In the present study, the variation of the feast/famine ratio with the increase of OLR did not affect the macroscopic structure of aerobic granules because it was kept lower than 0.24 during Stage I. However it could be the cause of the limitation in the formation of new aerobic granules during Stage II since the feast/famine ratio was of 0.38 on day 270.

5.4.3. Nitrogen removal

The applied nitrogen loading rate was stepwise increased from 0.39 kg N/(m^3 d) up to a value of 1.26 kg N/(m^3 d) on day 108 (S. ID) (Table 5.1). During the first days of operation, the ammonia removal was almost inexistent, but after 23 days from start up, a decrease of ammonia concentration was detected and both nitrite and nitrate were measured in the effluent. The increase of applied OLR and NLR was correlated with an increase of nitrogen removal efficiency which was maintained in values around 54%, 67% and 70% in stages S. IA, S. IB and S. IC, respectively. However, the application of a loading rate of 1.26 kg N/(m^3 d) (S. ID) led to a high variability in the removal efficiency of the system as it is shown in Figure 5.11.

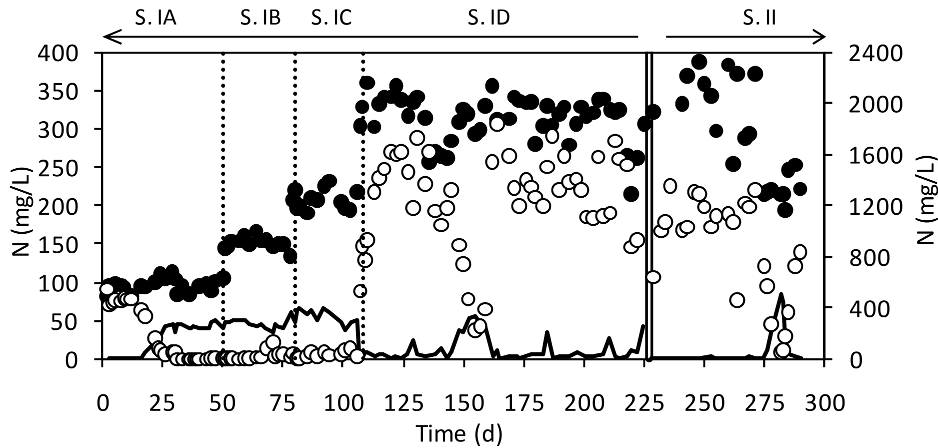


Figure 5.11. Concentrations (mg N/L) of NH_4^+ in the influent (●) and NH_4^+ (○) and $NO_x^- = NO_2^- + NO_3^-$ (—) in the effluent of the SBR.

Nitrogen balances were calculated to the reactor in order to determine the amount of nitrogen used for bacterial growth and to check if the nitrogen removal was mainly due to denitrification or biomass assimilation (Table 5.2). Periods of stable operation in terms of nitrogen compounds concentration in stages S. IA, S. IB and S. IC were selected and the calculations were based in the biomass growth as indicated in Chapter 2. As an example, during stage S. IA the 41% of the nitrogen removed was used for bacterial growth, and the rest was removed via nitrification-denitrification. With the increase of the NLR the amount of nitrogen removed using the second via increased.

Table 5.2. Nitrogen mass balances during different operational periods.

Stage	Days	$N_{removed}$ [a] (mg/d)	$N_{assimilated}$ [b] (mg/d)	Nitrogen removal efficiency (%)		
S. IA	24 - 50	312	128	54	$N_{assimilated}$ (%)	41
					$N_{denitrified}$ (%)	59
S. IB	51-79	606	109	67	$N_{assimilated}$ (%)	18
					$N_{denitrified}$ (%)	72
S. IC	80-105	888	200	70	$N_{assimilated}$ (%)	22
					$N_{denitrified}$ (%)	78

[a]Calculated as the difference between the influent and the effluent nitrogen content, [b]calculated from the biomass growth

The nitrogen removal efficiency during stages S. ID and S. II was variable, with a period of only 15 days (days 145-160) in which the nitrogen removal seemed to recover to previous

values (Figure 5.11). In the remaining days of both stages nitrogen was mainly removed due to biomass production.

In order to better understand the evolution of the nitrogen compounds, cycle measurements were performed during the different stages. In the analysis performed on day 44 (Figure 5.12.A) the decrease of nitrogen oxides concentrations in the bulk liquid was observed during the first 15 min of operation due to the occurrence of the denitrification process. During the famine phase the ammonia was mainly oxidized to nitrate during the first 120 minutes, as it was previously observed by Mosquera-Corral *et al.*, (2005).

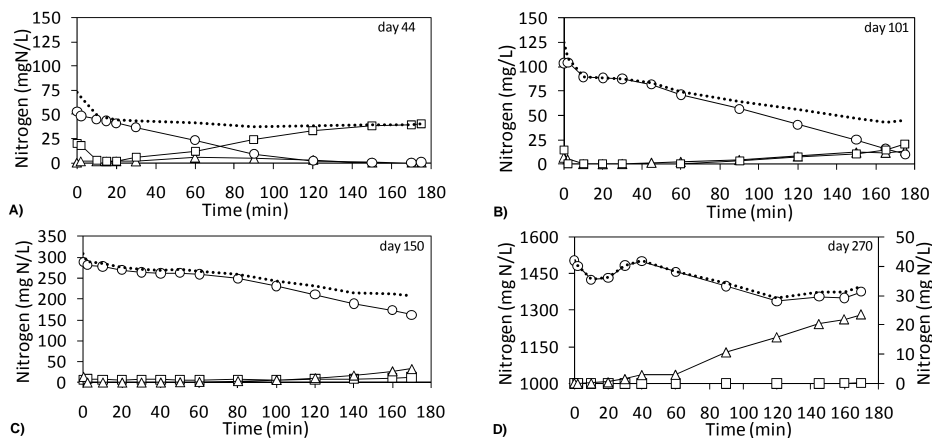


Figure 5.12. Evolution of NH_4^+-N (○), NO_2^--N (△), NO_3^--N (□) and total nitrogen (…) concentrations (mg N/L) during one operational cycle in operational days A) 44 (S. IA), B) 101 (S. IC), C) 150 (S. ID) and D) 270 (S. II).

The profiles on day 101 and day 150 were different from the previous one (Figure 5.12 B) and C)). In both cases the simultaneous nitrification and denitrification (SND) process was observed. This is a common phenomenon in aerobic granular systems due to the presence of aerobic and anoxic zones in the granule and has been demonstrated either using synthetic or industrial wastewater (Zeng *et al.*, 2003; de Kreuk *et al.*, 2005; Yilmaz *et al.*, 2008).

Furthermore, this denitrification occurs in absence of organic matter in the liquid media (famine phase) due to the presence of polyhydroxyalkanoates (PHA) pre-accumulated in the biomass that could be utilized for cell maintenance and denitrification in the inner zones of the granule when external carbon source is absent (Third *et al.*, 2003; Qin *et al.*, 2005; Adav *et al.*, 2009).

From the data obtained in the cycle performed on day 101 (S. IC), 94 mg N/L of the fed ammonia were removed along all the length of the cycle, with the accumulation of less than 21 and 25 mg N/L of nitrite and nitrate, respectively. In the present research, denitrification

was carried out in spite of the long aeration phase. Although the measurement of the dissolved oxygen in the bulk liquid on day 101 indicated that the level was around 4.0 mg O_2/L during the famine phase (Figure 5.10). The oxygen mass transfer limitations from the liquid to the granule and its consumption by microorganisms cause the decrease of the oxygen concentration in the inner layer of the granules (Rasmussen and Lewandowski, 1998).

In those cases when nitrification and denitrification processes occur under low dissolved oxygen concentration and rapid changes of the operational conditions are performed, the emission of nitrogen oxides and methane to the gas phase can be produced (Kampschreur *et al.*, 2009). Punctual measurements of gas samples on days 80, 101 and 220 of NO_x during the feast phase did not show the presence of these compounds in the head space of the reactor.

Nitrogen removal efficiencies and consumption and production rates (Table 5.3) were calculated during the four analyzed cycles as described in Chapter 2. Even though removal efficiencies were similar between days 44 and 101, a close look to the data reveals that ammonia specific consumption rate is almost double on day 44 while nitrate production rate is four- fold lower. The difference in the solids concentration in the reactor caused that the nitrogen consumption rate in both days were similar. On day 150, with a similar solids concentration as on day 101, the ammonia and nitrogen consumption rates were similar to day 44.

Table 5.3. Summary of the results obtained in each one of the different cycles.

Parameter	Stage	Day 44 S. IA	Day 101 S. IC	Day 150 S. ID	Day 270 S. II
DO concentration (feast/famine)		2.0/6.5	2.3/4.0	1.4/5.0	1.2/6.4
Biomass concentration (g VSS/L)		5.0	11.0	11.5	10.0
Removal efficiency of nitrogen (%)		62	73	44	27
Maximal ammonia specific consumption rate (mg NH_4^+-N/(g VSS d))		131.6	69.4	118.9	153.5
Maximal nitrite specific production rate (mg NO_2^--N/(g VSS d))		12.1	14.0	32.4	27.1
Maximal nitrate specific production rate (mg NO_3^--N/(g VSS d))		91.2	12.3	9.8	1.8
Nitrogen removed during the cycle (mg N/L)		32.5	71.7	109.2	151.4
Ammonia consumed (mg NH_4^+ -N/L)		52.5	94.2	135.8	151.5
Nitrite and nitrate produced (mg N/L)		37.1	46.2	37.6	23.0
COD removed (mg COD/L)		295.3	496.2	745.6	597.3
Nitrogen used for growth (mg N/L)*		6.7	11.3	16.9	13.6
COD/N ratio		5.8	5.4	5.5	7.6

*Supposing 0.19 g VSS/g COD and 0.12 g N/g VSS

The amount of nitrogen consumed for growth in each cycle was calculated from the COD removed in the cycle in order to minimize the experimental errors associated with the solids concentration determination at the beginning and at the end of the cycle. A comparison of the present results with those obtained in Chapter 3 (Table 3.5) indicated that the difference between the nitrogen removed and the nitrogen assimilated for each system is noticeable. In Chapter 3 the amount of nitrogen assimilated ranged between 34% and 100% of the nitrogen removed whereas in the present work the percentage calculated for each cycle ranged from 9% to 20%. Higher ammonia concentrations in the reactor with longer feast/famine periods would favour the simultaneous nitrification and denitrification in the present reactor and would explain such differences in nitrogen removal.

During stages S. ID and S.II the nitrification was variable, and the same factors that were reported in Chapter 3 to affect the nitrifying activity in the aerobic granular reactor can be applied to this system. Among others, during stage S. ID the free ammonia concentration in the system increased. The pH values ranged from 8.3 during the feast phase to 7.9 at the end of each measured cycle, which correlated to the ammonia concentration produced high free ammonia concentration. The results obtained from the operational cycle measured on day 150, indicated that free ammonia concentrations were higher than 20 mg N/L during more than 160 min.

The increase of OLR could also cause the decrease of granule nitrifying activity due to the reduction of oxygen penetration depth in the granules surface and the decrease of dissolved oxygen available for ammonia oxidizing bacteria (Chen et al., 2008). The applied OLR also promoted the faster growth of heterotrophic bacteria, characterized by higher growth rate than ammonia oxidizing bacteria.

5.5. CONCLUSIONS

Aerobic granular sludge technology showed its feasibility for the treatment of swine slurries in an SBR reactor operated for more than 300 days. Aerobic granules showed excellent settling properties (SVI of 32 mL/g TSS and density of 55 g VSS/$L_{granule}$ on day 140) and the granular structure was maintained during all the operational period (feret diameter that ranged between 3.0 and 5.2 mm) in spite of a granular breakage event.

The system treated simultaneously an OLR of 4.4 kg COD/(m^3 d) and NLR of 0.83 kg N/(m^3 d) with organic matter and nitrogen removal efficiencies up to 87% and 70%, respectively. Not complete organic matter removal was achieved due to a fraction of 10% of presumably slowly or not biodegradable COD. Substrate profiles during an operational cycle when the system was operated under these conditions showed that simultaneous nitrification and denitrification (SND) was achieved. When the loading rate increased and the OLR and NLR treated reached values of 7.0 and 1.26 kg N/(m^3 d), respectively the decrease of the nitrogen removal efficiency was observed.

The reduction of volumetric exchange ratio from 50% to 6% for the treatment of raw slurry did not affect the reactor performance in terms of organic matter. Values of OLR up to 7.3 kg COD/(m^3 d) were treated in the system without loose of the organic matter removal efficiency although the nitrogen removal via nitrification-denitrification disappeared. Biomass characteristics changed significantly, due to the increase of solids concentration in the influent up to 4.9 g VSS/L and the growth of dispersed biomass. Biomass concentration in the reactor decreased from 16.0 to 4.7 g VSS/L because granules were trapped in that dispersed biomass during the effluent withdrawal.

5.6. REFERENCES

Adav S., Lee D.J. and Lai J.Y. (2009). Biological nitrification–denitrification with alternating oxic and anoxic operations using aerobic granules. *Applied Microbiology and Biotechnology,* 84(6), 1181-1189.

An J.Y., Kwon J.C., Ahn D.W., Shin D.H., Shin H.S. and Kim B.W. (2007). Efficient nitrogen removal in a pilot system based on upflow multi-layer bioreactor for treatment of strong nitrogenous swine wastewater. *Process Biochemistry,* 42(5), 764-772.

APHA-AWWA-WPCF (2005) Standard methods for the examination of water and wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Beline F., Daumer M.L., Loyon L., Pourcher A.M., Dabert P., Guiziou F. and Peu P. (2008). The efficiency of biological aerobic treatment of piggery wastewater to control nitrogen, phosphorus, pathogen and gas emissions. *Water Science and Technology,* 57(12), 1909-1914.

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(3), 702-712.

Chen Y., Jiang W., Liang D. and Tay J. (2008). Biodegradation and kinetics of aerobic granules under high organic loading rates in sequencing batch reactor. *Applied Microbiology and Biotechnology,* 79(2), 301-308.

Cheng J. and Liu B. (2001). Nitrification/Denitrification in intermittent aeration process for swine wastewater treatment. *Journal of Environmental Engineering,* 127(8), 705-711.

de Godos I., Gonzalez C., Becares E., Garcia-Encina P.A. and Munoz R. (2009). Simultaneous nutrients and carbon removal during pretreated swine slurry degradation in a tubular biofilm photobioreactor. *Applied Microbiology and Biotechnology,* 82(1), 187-194.

de Kreuk M., Heijnen J.J. and van Loosdrecht M.C.M. (2005). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnology and Bioengineering,* 90(6), 761-769.

de Kreuk M.K., Kishida N., Tsuneda S. and van Loosdrecht M.C.M. (2010). Behavior of polymeric substrates in an aerobic granular sludge system. *Water Research,* 44(20), 5929-5938.

Deng L.-W., Zheng P. and Chen Z.-A. (2006). Anaerobic digestion and post-treatment of swine wastewater using IC-SBR process with bypass of raw wastewater. *Process Biochemistry,* 41(4), 965-969.

European Parliament (2008). European Parliament, Committee on Agricultural and Rural Development. Report on Sustainable Agriculture and Biogas: a need for review of EU Legislation (2007/2107(INI)).

FAOSTAT (2009) Agricultural production data (Live animals). Web page: http://faostat.fao.org/site/573/DesktopDefault.aspx?PageID=573#ancor

Han Z., Wu W., Ding Y., Zhu J. and Chen Y. (2008). Optimization of main factors associated with nitrogen removal in hybrid sludge sequencing batch reactor with step-feeding of swine wastewater. *Journal of Environmental Science and Health, Part A,* 43(2), 161-170.

Kampschreur M.J., Temmink H., Kleerebezem R., Jetten M.S.M. and van Loosdrecht M.C.M. (2009). Nitrous oxide emission during wastewater treatment. *Water Research,* 43(17), 4093-4103.

Lampert C. (2003). Selected requirements on a sustainable nutrient management. *Water Science and Technology,* 48(1), 147-154.

Lemaire R., Webb R.I. and Yuan Z. (2008). Micro-scale observations of the structure of aerobic microbial granules used for the treatment of nutrient-rich industrial wastewater. *The ISME Journal,* 2(5), 528-541.

Liu Y.Q. and Tay J.H. (2007). Characteristics and stability of aerobic granules cultivated with different starvation time. *Applied Microbiology and Biotechnology,* 75(1), 205-210.

Mace S. and Mata-Alvarez J. (2002). Utilization of SBR technology for wastewater treatment: An overview. *Industrial & Engineering Chemistry Research,* 41(23), 5539-5553.

Magrí A. and Flotats X. (2008). Modelling of biological nitrogen removal from the liquid fraction of pig slurry in a sequencing batch reactor. *Biosystems Engineering,* 101(2), 239-259.

McSwain B.S., Irvine R.L. and Wilderer P.A. (2004). Effect of intermittent feeding on aerobic granule structure. *Water Science and Technology,* 49(11-12), 19-25.

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.

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(6), 407-412.

Obaja D., Macé S. and Mata-Alvarez J. (2005). Biological nutrient removal by a sequencing batch reactor (SBR) using an internal organic carbon source in digested piggery wastewater. *Bioresource Technology,* 96(1), 7-14.

Qin L., Liu Y. and Tay J.H. (2005). Denitrification on poly-[beta]-hydroxybutyrate in microbial granular sludge sequencing batch reactor. *Water Research,* 39(8), 1503-1510.

Rasmussen K. and Lewandowski Z. (1998). Microelectrode measurements of local mass transport rates in heterogeneous biofilms. *Biotechnology and Bioengineering,* 59(3), 302-309.

Schwarzenbeck N., Borges J.M. and Wilderer P.A. (2005). Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor. *Applied Microbiology and Biotechnology,* 66(6), 711-718.

Shin J.H., Lee S.M., Jung J.Y., Chung Y.C. and Noh S.H. (2005). Enhanced COD and nitrogen removals for the treatment of swine wastewater by combining submerged membrane bioreactor (MBR) and anaerobic upflow bed filter (AUBF) reactor. *Process Biochemistry,* 40(12), 3769-3776.

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

Su J.-J., Kung C.-M., Lin J., Lian W.-C. and Wu J.-F. (1997). Utilization of sequencing batch reactor for *in situ* piggery wastewater treatment. *Journal of Environmental Science and Health . Part A: Environmental Science and Engineering and Toxicology,* 32(2), 391 - 405.

Third K.A., Burnett N. and Cord-Ruwisch R. (2003). Simultaneous nitrification and denitrification using stored substrate (PHB) as the electron donor in an SBR. *Biotechnology and Bioengineering,* 83(6), 706-720.

Tijhuis L., van Benthum W.A.J., van Loosdrecht M.C.M. and Heijnen J.J. (1994). Solids retention time in spherical biofilms in a biofilm airlift suspension reactor. *Biotechnology and Bioengineering,* 44(8), 867-879.

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.

Yilmaz G., Lemaire R., Keller J. and Yuan Z. (2008). Simultaneous nitrification, denitrification, and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. *Biotechnology and Bioengineering,* 100(3), 529-541.

Zeng R.J., Lemaire R., Yuan Z. and Keller J. (2003). Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering,* 84(2), 170-178.

Zheng Y.M., Yu H.Q., Liu S.H. and Liu X.Z. (2006). Formation and instability of aerobic granules under high organic loading conditions. *Chemosphere,* 63(10), 1791-1800.

Chapter 6:

Evaluation of the CANON process for nitrogen removal from pre-treated swine slurry

Summary

The use of the completely autotrophic nitrogen removal over nitrite (CANON) process as a post-treatment for nitrogen removal from pre-treated slurry was studied in the present work. The ammonium removal under oxygen-limited conditions was researched in an air pulsing reactor operated at room temperature. In this system anammox bacteria grew mainly in the form of granules with an average feret diameter of 2.6 mm, and aerobic ammonium oxidizing bacteria mainly as dispersed biomass. The achieved nitrogen removal rate was of 0.46 kg N/(m^3 d) treating 300 mg NH_4^+-N/L with values of nitrogen removal efficiencies around 75 %.

The presence of organic matter at concentrations of 500 mg COD/L did not affect the operation of the process since the biomass grew in two different phases (granular and suspended) and this minimized the competition of microorganisms for the common substrates of ammonia oxidizing, anammox and heterotrophic bacteria.

The comparison of this aerobic process with other post-treatments for effluents from anaerobic digesters showed that the CANON process is a promising alternative to remove nitrogen from effluents generated in pig farms.

Keywords: aerobic granular biomass; anammox; CANON; pulsing reactor; nitrogen; swine slurry, temperature.

6.1. INTRODUCTION

Energy policies of the European Union are turning to promote the treatment and energy recovery of wastes from the pig farms characterized by high pollutants concentrations. With these regulations, the aim of reducing emissions of volatile organic compounds, controlling odours, mineralizing nutrients, improving its fertilizing properties or recovering energy by biogas production can be achieved. However, a definitive technological solution suitable for all the possible scenarios in the management of swine manure does not exist, and different technological solutions have been proposed in the last years (Flotats *et al.*, 2009) based in physical, chemical or biological treatments.

The system chosen for a particular site is dependent on several aspects:

- Local climate
- Environmental constraints
- Final utilization site of nutrients
- Capital and operating costs
- Labour requirements
- Convenience
- Technical requirements

Generally, the separation of both solid and liquid fractions of swine manure can be used as a simple method to enhance the management capability, so, the first step will consist in the installation of a solid-liquid separator (Figure 6.1). The solid fraction obtained after this initial separation together with that generated in the biological processes can be composted and sold either as fertilizer or for direct land spreading, or used in a small anaerobic digester for biogas production (Møller *et al.*, 2004). The liquid fraction can be biologically processed in order to remove both COD and nitrogen. Phosphorus recovery through chemical precipitation of phosphates (Yoshino *et al.*, 2003) is also considered in order to reduce the amount of phosphorus spread on land. For this purpose it is recommended to use the liquid fraction of anaerobic digesters.

Two options are possible for the biological treatment of the liquid fraction: aerobic and anaerobic processes (Bernet and Béline, 2009).

Aerobic treatment can be very effective in dealing with a wide range of pollution problems from swine slurry (organic matter, nitrogen, pathogens), but it is often not popular due to the high energy costs implied (Burton, 1992). In these systems ammonia nitrogen can be removed biologically via the conventional nitrification-denitrification process based on a sequence of aerobic and anoxic steps or via simultaneous nitrification-denitrification as seen in Chapter 5. Sustainable solutions regarding nitrogen removal from swine effluents are

under study based in biological aerobic processes (Cheng and Liu, 2001; An *et al.*, 2007; Loughrin *et al.*, 2009; Figueroa *et al.*, 2011).

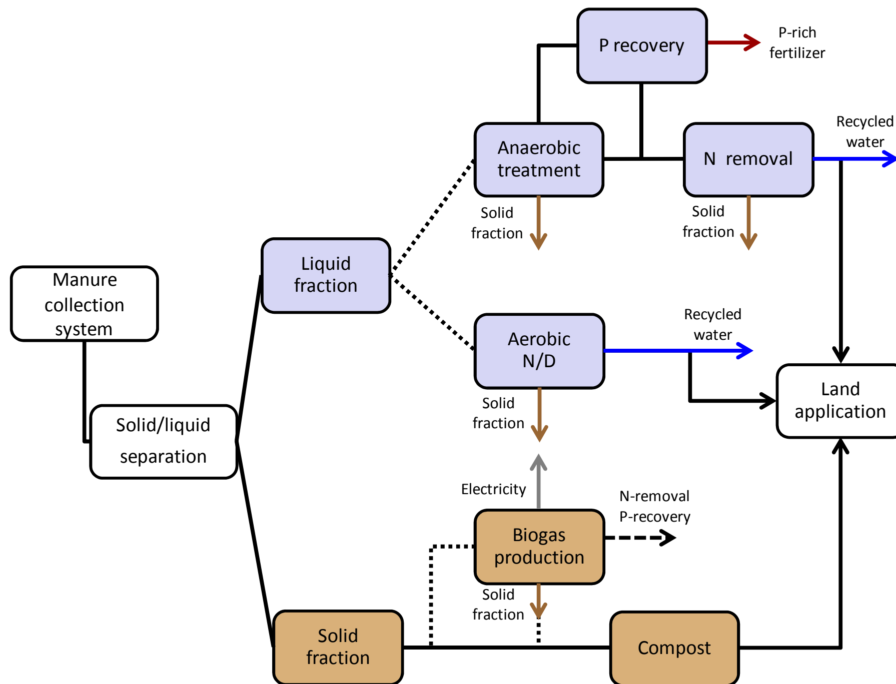


Figure 6.1. Some options available for effluent treatment at a piggery farm. The anaerobic and aerobic alternatives for the treatment of the liquid fraction are indicated. The sludge generated in the biological processes would be redirected to the treatment option considered for the solid fraction.

The use of an anaerobic treatment, widely extended in swine farms, allows the removal of organic matter with relatively low sludge production and low energy requirements (Holm-Nielsen *et al.*, 2009). This process can be carried out either in deep earthen outdoor basins (anaerobic lagoons covered for green house gas emissions recovery) or in reactor systems operated at mesophilic conditions. In this sense, technologies such as continuously stirred tank reactors (CSTR), plug flow or Upflow Anaerobic Sludge Blanket (UASB) reactors, were applied, working with OLR between 0.2-5.0 kg COD/(m^3 d) with high COD removal efficiencies (40-80%). The drawbacks of anaerobic process are the necessity of long retention times (20±30 days or longer) to achieve high removal efficiencies and that the organic nitrogenous compounds contained in the waste, such as proteins, aminoacids or urea, are mainly released as ammonia which is not further degraded in anaerobic conditions.

Currently, the European Nitrate Directive (EC/91/676) and the Spanish legislation (Real Decreto 261/1996) have established the maximum load of nitrogen to be spread by year on lands as 170 kg of nitrogen per hectare. Since the amount of land to spread the N-rich

effluent generated in anaerobic digesters is limited it is necessary to establish a post-treatment for nitrogen removal (Burton, 1992). In this way, the percentage of reduction of nitrogen content is the percentage of reduction of the acreage necessary if the load limits are not exceeded.

The effluent of the anaerobic reactor could be treated by recent technologies such as an anammox system. Since during the anammox process nitrite is used as electron acceptor to oxidize ammonia into nitrogen gas, a previous partial nitritation process, where 50% of the ammonia content is oxidized into nitrite, is needed. Both processes can be performed using two different reactors (e.g. SHARON process for partial nitritation plus anammox) or using a single reactor by means of the so called CANON process.

The feasibility of the anammox process for nitrogen removal has already been tested at industrial scale for different effluents (Abma *et al.*, 2007), however, no references for the post-treatment of the liquid fraction from anaerobic digesters treating livestock streams at industrial scale are available. Up to now this technology has been only applied at lab-scale using the configuration of partial nitritation and anammox in a two-unit configuration at temperatures that ranged between 30-40 °C (Ahn *et al.*, 2004; Hwang *et al.*, 2005; Furukawa *et al.*, 2009), since the optimal temperature for anammox bacteria is around 35 °C (Strous *et al.*, 1999). Albeit, it has been recently shown that the anammox and also the CANON processes could be operated at temperatures around 20 °C (Yang *et al.*, ; Isaka *et al.*, 2007; Dosta *et al.*, 2008; Vázquez-Padín *et al.*, 2009a; Vázquez-Padín *et al.*, 2009b).

This fact opens the possibility of applying this technology for the treatment of swine slurry at moderate temperature (e.g. as a post-treatment of the effluents from anaerobic lagoons).

6.2. OBJECTIVE

The objective of this work was to study the feasibility of the application of the CANON process as a post-treatment to remove nitrogen from pre-treated swine slurry, (in this case the effluent from the aerobic granular reactor presented in Chapter 5). The CANON process took place in an air pulsing sequencing batch reactor (SBRP), operated at room temperature, and its operation was evaluated in terms of nitrogen removal efficiency paying special attention to the characteristics of the formed biomass.

6.3. MATERIALS AND METHODS

6.3.1. Reactor description

A laboratory scale sequencing batch air pulsing reactor (SBRP) with a working volume of 1.5 L and a height to diameter ratio of 5.5 was used. Dissolved oxygen was supplied by a diaphragm pump (Laboport N86, KNF) with an electrovalve that controlled the pass of air at a

constant frequency of 0.09 1/s, meaning that air was supplied in repeated cycles of one second of flow and 10 seconds without flow. The air entered the system through an air sparger located at the bottom of the reactor, which provided good mixture inside the reactor and maintained suitable dissolved oxygen (DO) concentration to carry out the CANON process.

The volumetric exchange ratio was fixed at 50%. A set of two peristaltic pumps was used to introduce the feeding solution (on top of the reactor) and to discharge the effluent (at medium height in the column reactor), respectively. A programmable logic controller (PLC) (S7-224CPU, Siemens) controlled the actuations of the pumps and valves and regulated the different periods of the operational cycle (Figure 6.2A).

6.3.2. Operational conditions and feeding composition

The reactor was operated in cycles of 360 minutes distributed as follows: 345 minutes of aerated feeding, 10 minutes of settling and 5 minutes of effluent withdrawal (Figure 6.2B). The hydraulic retention time was fixed at 0.5 d with a feeding flow rate of 2.18 mL/min.

The SBRP was operated at room temperature (18 - 24 °C) and without pH control. The pH value inside the reactor was 7.7 ± 0.2. The DO concentration (Oxi 330i, WTW) ranged between 0.2 and 0.4 mg O_2/L along the operational period and was regulated by changing the air volume injected in each pulse (between 2.0 and 4.2 L/min) and keeping the frequency of pulsation constant.

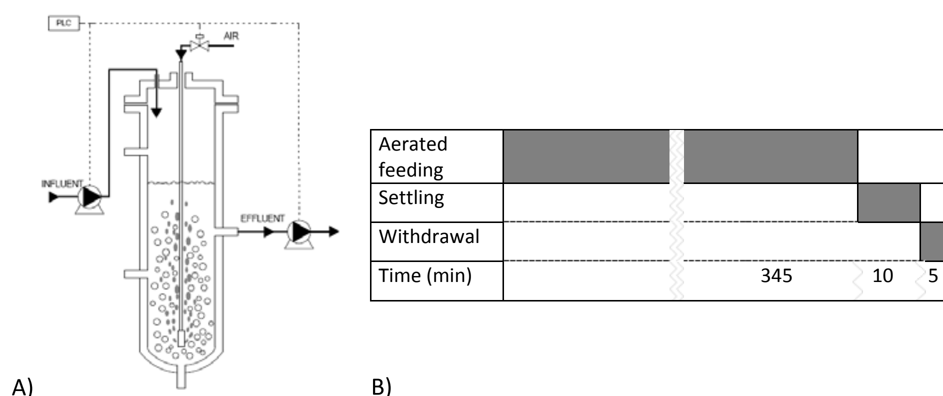


Figure 6.2. Experimental set-up (A) and distribution of the operational cycles (B) of the pulsing SBRP reactor.

In a previous work the SBRP was fed during 225 days with the effluent from the anaerobic sludge digester in operation at the urban wastewater treatment plant of Lugo (Spain); the operational data and conditions are gathered in Vázquez-Padín *et al.*, 2009b. After this date the influent composition was changed (indicated as day 0 in the figures) and

the system was fed with diluted swine slurry previously treated in an aerobic granular sludge reactor where the organic matter was removed (Chapter 5, Figueroa *et al.*, 2011). This pre-treated swine slurry was collected daily and kept refrigerated at 4 °C. The composition of the pre-treated swine slurry varied according to the performance of the aerobic granular reactor. In terms of nitrogen and organic matter the composition was as follows: 245 ± 16 mg NH_4^+-N/L, 4.1 ± 1.2 mg NO_2^--N/L, 3.3 ± 0.8 mg NO_3^--N/L and 420 ± 60 mg COD/L.

6.3.3. Analytical methods

Analytical determination of total suspended solids (TSS), volatile suspended solids (VSS), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), conductivity and pH was carried out according to Standard Methods (APHA-AWWA-WPCF, 2005). Concentrations of total organic carbon (TOC) and inorganic carbon (IC) were measured with an analyser (TOC-5000, Shimadzu). Chemical Oxygen Demand (COD) soluble (COD_s) or total (COD_t) was determined by a semi-micro method (Soto *et al.*, 1989).The morphology and size distribution of the granules were measured regularly by using an image analysis procedure (Tijhuis *et al.*, 1994) with a stereomicroscope (Stemi 2000-C, Zeiss).

6.4. RESULTS AND DISCUSSION

6.4.1. Nitrogen removal

A conservative operational strategy was applied to introduce the pre-treated swine slurry as feeding media and avoid the inhibition events of the anammox bacteria by oxygen and/or nitrite concentrations (Dapena-Mora *et al.*, 2007). Therefore, the efficiency of this CANON system was always limited by the amount of air supplied and the dissolved oxygen concentration in the bulk liquid was maintained around 0.3 mg O_2/L. The pulsed air volume was used as a control parameter to maintain the suitable DO concentration in a similar way as proposed by Wett (2006). Moreover, the application of a pulsating flow is expected to decrease the aeration costs compared with a continuously aerated CANON system (Vázquez-Padín *et al.*, 2009a).

In those cases when ammonia was left in the effluent and since nitrite concentration (lower than 4 mg NO_2^--N/L) was far away from the inhibitory levels for this compound (30-150 mg NO_2^--N/L (Fernández *et al.*, 2011)), the pulsed air volume was increased to augment the ammonia oxidation activity. This strategy did not cause an increase of DO and nitrite concentrations in the liquid media because of its fast consumption by ammonia oxidizing bacteria (AOB) and anammox bacteria, respectively, but it raised the nitrogen removal rate. In this way the ammonia concentration in the effluent was reduced with the increase of the air flow rate in the air pulses (Figure 6.3).

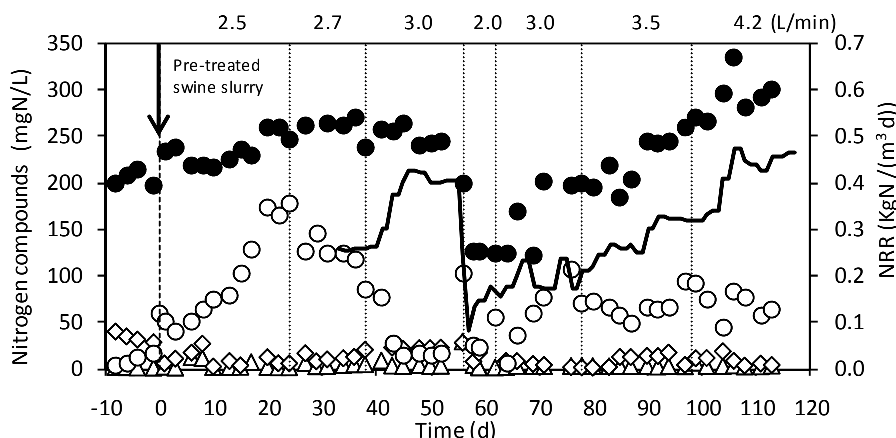


Figure 6.3. Evolution of nitrogen compounds in the CANON reactor: NH_4^+ in the influent (●), and NH_4^+ (○), NO_2^- (△) and NO_3^- (◇) in the effluent. Nitrogen removal rate in the reactor (—). Dotted lines (…) indicate a change in the flow of pulsed air.

Ammonia concentration in the influent of the CANON system was around 250 mg N/L and the nitrogen removal rate (NRR) was of 0.41 kg N/(m^3 d) around day 50 of operation. At this point, the ammonia concentration in the influent was of 241 ± 3 mg NH_4^+-N/L and in the effluent the concentrations of the nitrogen compounds were of 16.2 ± 1.0 mg NH_4^+-N/L, 2.5 ± 0.3 mg NO_2^--N/L and 22.3 ± 0.3 mg NO_3^--N/L. Hence, the removal efficiencies were of 93 % and 83 % as ammonia and total nitrogen, respectively.

A failure in the aeration valve on day 56 caused an increase of the dissolved oxygen concentration up to 5 mg O_2/L. This caused the inhibition of the anammox bacteria and, therefore, the accumulation of nitrite (30 mg NO_2^--N/L) inside the reactor. In order to recover the system efficiency the ammonia concentration, by proper dilution with tap water during 20 days, and the volume of the pulsed air during 6 days were diminished. After 30 days the nitrogen load applied to the system before the destabilization was restored, and the nitrogen removal capacity increased continuously until the end of the experiment.

At the end of the operational period, the NRR was around 0.46 kg N/(m^3 d) when treating 300 mg NH_4^+-N/L, with nitrite and nitrate concentrations in the effluent lower than 3 and 10 mg N/L, respectively. The removal efficiencies were of 80 % and 77 % as ammonia and total nitrogen, respectively. The nitrogen removal rate was equal to that achieved by this system treating the effluent of an anaerobic digester and operated at room temperature (Vázquez-Padín *et al.*, 2009b). These values were in the range of 0.06 – 1.50 kg N/(m^3 d) reported for different CANON systems operated at temperatures around 30 ºC (Vázquez-Padín *et al.*, 2009b).

The use of a pulsating flow would reduce the energy requirements for aeration and would allow an easy control of the dissolved oxygen level by changing the pulsating frequency or the amount of air pulsed.

6.4.2. Presence of organic matter

The effect of organic matter on the performance of the anammox process is a little bit controversial. Authors like de Graaf *et al.*, (1996) found that organic compounds such as acetate, propionate, glucose, fructose or lactose did not cause significant decrease of activity or even increased the anammox activity when they were added to batch tests. The former effect occurred for example with concentrations of sodium acetate trihydrate up to 385 mg COD/L. However, during experiments in continuous operation, organic compounds can favour the growth of heterotrophic denitrifying bacteria which compete with anammox bacteria for nitrite (Kumar and Lin, 2010). Since the growth rate of heterotrophic denitrifying bacteria is faster than that of anammox bacteria, this competition for nitrite is generally won by the denitrifiers ones while the anammox activity is gradually lost (Kumar and Lin, 2010). Organic matter content around 300 mg COD/L or inlet COD to N ratio of over 2.0 g COD/g N were found to cause severe failure in anammox communities under anaerobic conditions (Chamchoi *et al.*, 2008; Molinuevo *et al.*, 2009). However certain species of Anammox bacteria have been observed to be able to grow on organic matter and compete successfully with heterotrophic denitrifiers (Güven *et al.*, 2005; Kartal *et al.*, 2008).

Information regarding the behaviour of CANON systems exposed to COD concentrations in the treated influent is scarce, and it has been studied mainly via mathematical simulations (Hao and van Loosdrecht, 2004; Lackner *et al.*, 2008). In both previous studies anaerobic ammonia oxidation capacity could be maintained in spite of the use of COD to N ratios of over 2.0 g COD/g N. It must be taken into account that the CANON system is operated under microaerobic conditions and that the most favourable process for COD removal would be the use of oxygen as electron acceptor for its oxidation. Then, in this case, a competition for oxygen between both heterotrophic bacteria and ammonia oxidizing bacteria would be established. Mosquera-Corral *et al.*, (2005) operated a SHARON system with a mixture of sodium acetate and ammonia and found that an inlet COD/N ratio of 0.6 g COD/g N caused a decrease of nitrifying activity. Nevertheless, Furukawa *et al.*, (2009) found that a partial nitritation system fed with digester liquor was able to maintain its nitrifying activity even when a ratio of 1.6 g COD/g N was applied. In the present work, the presence of organic matter did not cause a negative effect on the nitrogen removal performance of the CANON system even though an inlet COD/N ratio that ranged between 2.4 and 0.5 g COD/g N was applied. The different results from those reported in previous studies could be explained in this case by the use of pre-treated influents where most part of the organic matter consisted in non- or slowly-biodegradable COD. Therefore, the biodegradable organic fraction of the influent was different if an easily biodegradable substrate as acetate is used. Furthermore in

terms of comparison, to provide the organic matter in terms of COD that is removed by the system ($COD_{removed}$) is more important than the COD applied to the system.

In the present case, the operation of the CANON reactor was characterized by the fluctuations of the fraction of degradable organic matter in the influent. The value of $COD_{removed}$/N ratio ranged between 0.8-1.3 g $COD_{removed}$/g N until day 38 of operation, then, with the decrease of organic matter content in the feeding it was maintained below 0.6 g $COD_{removed}$/g N (Figure 6.4). During the operational period the applied ratios were lower than those obtained from the simulations to cause no significant effect on the performance of the CANON reactor, which confirm to a certain extent the simulated results (Hao and van Loosdrecht, 2004; Lackner *et al.*, 2008).

Besides the $COD_{removed}$/N ratio, the concentration of organic matter itself ($COD_{removed}$) in the system was considered due to the possible inhibitory effects reported in the case of enriched anammox systems (300 mg/L). In the present work, the value was around 250 mg $COD_{removed}$/L during the first days of operation and after day 38 it was around 60 mg $COD_{removed}$/L. The removed organic loading rate ($OLR_{removed}$) ranged between 0.1 and 0.6 kg COD/(m^3 d) with the COD removal efficiencies varying from 13% to 62%, respectively (Figure 6.4). As a consequence no effect of the present COD is expected when this kind of effluents are treated.

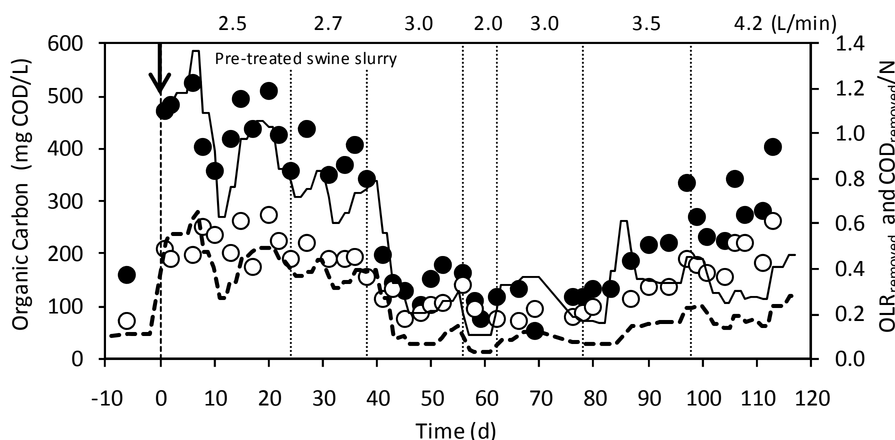


Figure 6.4. Evolution of organic matter concentration in the CANON reactor: COD in the influent (●) and in the effluent (○).Treated organic loading rate (– –) in the system (kg COD/m^3 d) and $COD_{removed}$/N ratio (g $COD_{removed}$/g NH_4^+-N) (—).

6.4.3. Biomass physical properties

At the beginning of the experiment the biomass concentration in the reactor was of 5.0 g VSS/L and the solids concentration in the effluent was of 0.011 g VSS/L which allowed working at a solids retention time (SRT) up to 279 d. With the application of the pre-treated

swine slurry, the solids concentration in the effluent increased up to over 0.15 g VSS/L during the first days of operation while the biomass concentration in the reactor increased, maybe due to the sudden growth of heterotrophic bacteria with worse settling properties. After day 20 of operation, the solids concentration inside the reactor was stabilized and maintained around 3.4 g VSS/L and the solids concentration in the effluent around 0.10 g VSS/L (Figure 6.5). The SRT decreased, and ranged from 15 to 40 d during all the operational period depending on the solids concentration in the effluent. Therefore, the minimum SRT of 7.9 d required to guarantee the anammox process (Park *et al.*, 2010) was achieved in the reactor.

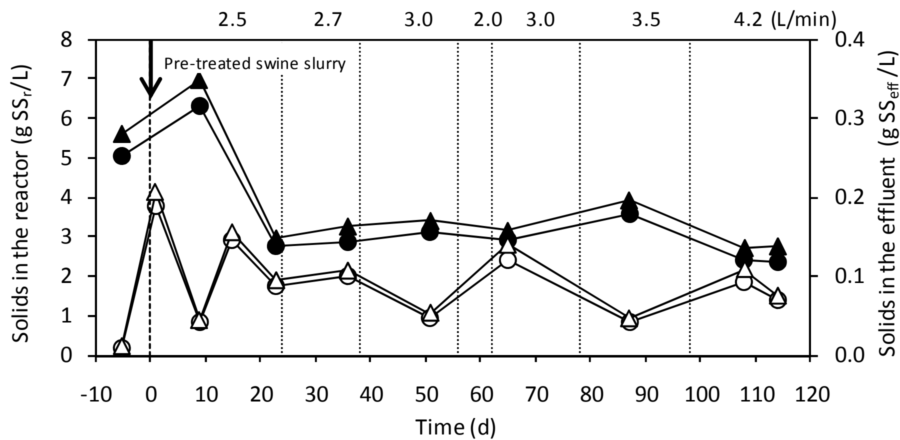


Figure 6.5. Solids content in the reactor: TSS (▲) and VSS (●); and in the effluent: TSS (△) and VSS (○) (g/L).

Initially the biomass was composed by granules with a mean diameter of 1.6 mm (Figure 6.6A). A significant change in the size of the granules was observed when the reactor was fed with the pre-treated swine slurry. On day 90 of operation the average feret diameter had reached values around 2.6 mm, with some granules of 5.6 mm of diameter (Figure 6.6 B) due to both the growth of new anammox granular biomass and the increase of the size of the already present granules.

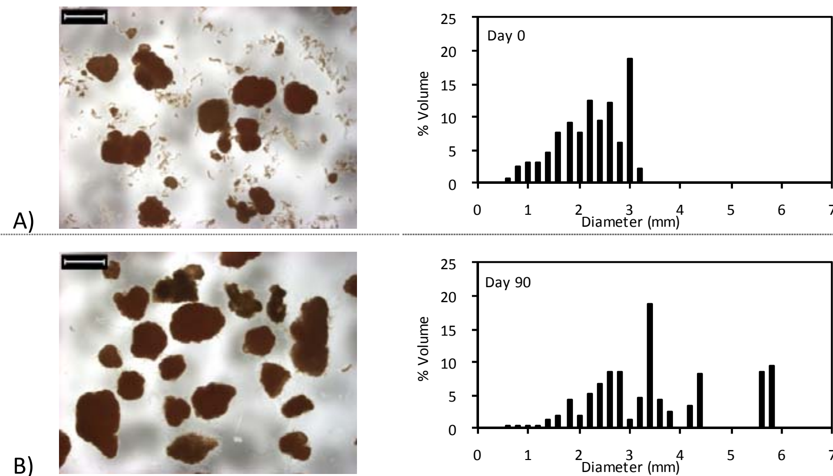


Figure 6.6. Image of the Anammox granules and their volumetric size distribution on days A) 0 and B) 90 of operation.

In this CANON system, the biomass was developed in two different states: granules mainly composed of anammox bacteria (identified by their reddish colour), and biomass in suspension, mainly composed of AOB and heterotrophic bacteria (Figure 6.7).

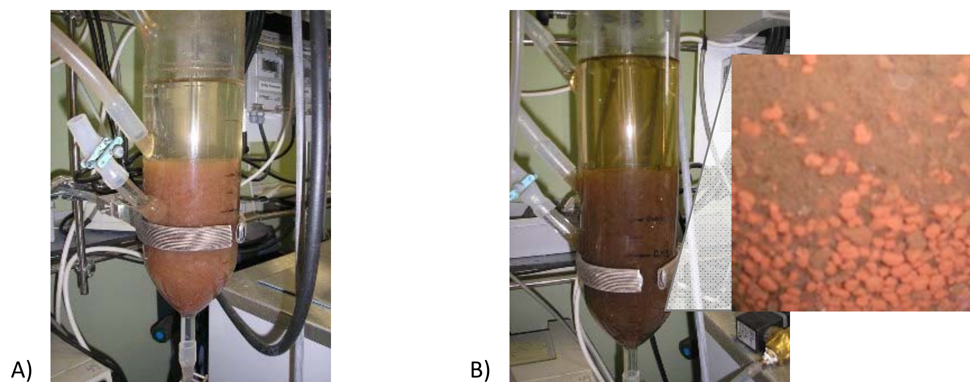


Figure 6.7. Images of the reactor A) prior to the change of the feeding (day -10) and B) during the new feeding (day 58).

The formation of this dispersed biomass could be a strategy of AOB to overcome substrate diffusion limitations due to the low DO concentrations in the bulk liquid and to outcompete heterotrophs (Mosquera-Corral *et al.*, 2005). Moreover, the growth of anammox bacteria mainly as granules and nitrifiers and heterotrophs mainly in suspension presents several advantages for all of them:

1. The anammox bacteria are protected from the oxygen, that causes inhibition, since the major part of the dissolved oxygen is consumed by the biomass in suspension and by a

thin layer of nitrifiers that covers the granule (Nielsen *et al.*, 2005). It must be pointed out that the DO concentration in the liquid media can be kept in lower levels than in the conventional CANON process where both populations grow in the granules.

2. Heterotrophs and nitrifiers will have easy access to the substrates in the liquid media.

3. The anammox bacteria in the granules are not diluted with heterotrophs. Excessive mixed growth of anammox, nitrifiers and heterotrophs in the granules will lead to dilution of the anammox, especially because of the large biomass yield difference between them (van Benthum *et al.*, 1997).

6.4.4. Evaluation of different strategies for swine slurry treatment

In order to determine the applicability of the developed CANON system as a post-treatment for an aerobic granular reactor to treat swine slurry a comparison with obtained results from another configurations and different processes has been performed.

The use of anaerobic digestion and a post-treatment based in a two-unit configuration for partial nitritation plus anammox was widely used (Table 6.1). This combination made it possible to remove nitrogen from swine slurry with high removal efficiencies, and to treat nitrogen loading rates up to 0.4 kg N/(m^3 d) when the reactor was operated at 35 °C (Hwang et al., 2006). In the research carried by Ahn et al. (2004) a NLR of 0.66 kg N/(m^3 d) with a total nitrogen removal efficiency of 80% was treated, but in this case, from 0.36 to 0.50 kg NO_2-N/(m^3 d) of nitrite had to be added to the influent in order to induce the anammox reaction. Some studies reflected problematic situations due to the presence of coagulants from the anaerobic step (Yamamoto *et al.*, 2008), residual biodegradable organic matter (Molinuevo *et al.*, 2009) or antibiotics (Fernandez *et al.*, 2009). The application of the two unit configuration would be appropriated in cases where inhibitory compounds are present since these compounds will be degraded in the nitrifying unit avoiding its entrance in the anammox reactor.

In the present study the pos-treatment was carried out in a single unit operated at moderate temperatures (20 °C) as an alternative for the nitrogen removal in those cases where the maintaining of high temperatures of operation in the reactor is not possible or the swine manure is not pre-treated at mesophilic conditions. The treated NLR by this system is in the range of other partial nitritation-anammox and CANON processes operated at high temperature either with synthetic media or industrial effluents, that ranged between 0.06 and 1.50 kg N /(m^3 d) (Vázquez-Padín *et al.*, 2009b). The use of a single unit system presents some advantages with respect to the two unit configuration such as, lower capital and aeration costs.

Another option for the treatment of swine slurry is the use of aerobic and anoxic processes such as nitrification-denitrification ones (Tilche *et al.*, 2001; Beline *et al.*, 2008). In

these cases to achieve the simultaneous COD and nitrogen removal treating high loading rates with high removal efficiencies is possible (Table 6.1).

Table 6.1. Comparison between different concepts for swine manure treatment.

	Ref.	Objective	Treated loading rates	Removal efficiencies	Temperature
ANAEROBIC PROCESS + POST-TRATAMENT	[1]	Organic matter, phosphorus and nitrogen removal via combination of anaerobic digestion elutriated phased treatment, SHARON and anaerobic ammonium oxidation (ADEPT-SHARON ANAMMOX)	4.9 kg COD /(m^3 d) (UASB) and 0.4 kg N /(m^3 d) (SHARON- Anammox)	COD: 88% NH_4^+-N: 94.1%, TKN: 86.5% PO_4-P: 72.2%	35 °C (UASB and anammox)
	[2]	Organic matter, nitrogen and phosphorus removal via combination of full-scale anaerobic digestion, decantation, UASB post-digestion, partial oxidation and OLAND process (PIGMAN)	1.1 kg COD /(m^3 d) (full scale plant); 3.25 kg COD /(m^3 d) (UASB) and 0.1 kg N /(m^3 d) (OLAND)	COD_s: 96% NH_4^+-N: 96%, Total N: 88% PO_4-P: 63%, Total P: 81%	55 °C (full scale and UASB) 35 °C (anammox)
	[3]	Organic matter and nitrogen removal in an upflow anaerobic sludge bed (UASB) reactor at mesophilic conditions, with addition of $NaNO_2$.	2.80 kg COD /(m^3 d) and 0.66 kg N /(m^3 d)	COD_s: 47-55% NH_4^+-N: 61.7%, Total N: 80%	35 °C
	[4]	Organic matter and nitrogen removal in a mesophilic digester. Partial nitritation and anammox for N removal in two different reactors.	1.0 kg N /(m^3 d) for partial nitritation and 0.22 kg N /(m^3 d) for anammox	COD: n.a. NH_4^+-N: 70%, Total N: 55%	35 °C (anaerobic digestion and anammox), 25 °C (partial nitritation)
	[5]	Organic matter and nitrogen removal in an anaerobic lagoon* and post-treatment in a CANON reactor	0.04-0.30* kg COD /(m^3 d) and 0.46 kg N /(m^3 d)	COD_s: 40-50%* NH_4^+-N: 78%, Total N: 75%	18 to 24 °C (CANON)
AEROBIC TREATMINT	[6]	Solids separation and simultaneous organic matter and nitrogen removal via nitrification/denitrification (full scale)	1.4 kg COD /(m^3 d) 0.25 kg N /(m^3 d)	COD: 96% TKN: 96%, NH_4^+-N: 97%	-2.5 to 31 °C
	[7]	Simultaneous organic matter and nitrogen removal via nitrification/denitrification with an aerobic granular sequencing batch reactor (SBR) (lab scale)	3.90 kg COD /(m^3 d) and 0.57 kg N /(m^3 d)	CODS: 88% NH_4^+-N: 97%, Total N: 70%	20 to 24 °C
	[8]	SBR with alternating oxic and anoxic conditions (cycle of 12 hrs anoxic, 10 hrs oxic and 2 hrs idling periods) (lab scale)	1.21 kg COD/ (m^3 d) and 0.186 kg TKN/ (m^3 d)	COD: n.a. TKN: n.a.	25 °C
	[8]	Solids separation, oxic and anoxic tanks and activated carbon (full scale)	1.0 to 1.5 kg COD/ (m^3 d) 0.1 to 0.2 kg TKN/ (m^3 d)	COD: n.a. TKN: n.a.	35 to 45 °C

[1] Hwang *et al.*, 2006 ; [2] Karakashev *et al.*, 2008; [3] Ahn *et al.*, 2004; [4] Yamamoto *et al.*, 2008; [5] This study; [6] Vanotti *et al.*, 2009; [7] Figueroa et al., 2011; [8] Choi and Eum, 2002. n.a. = data not avaliable.
*COD concentration extracted from EPA guidelines (EPA, 2002).

The main drawback of the systems based on aerobic treatment is that aeration requirements are higher than in those based on anaerobic processes, even if in the latter the post-treatment for nitrogen removal is included, and so they are the operational costs.

An estimation of the oxygen consumption of the biological process to remove 1 kg of NH_4^+-N can be performed for the different available technologies. In the case of a conventional nitrification-denitrification system the stoichiometric amount of consumed oxygen would be of 4.6 kg O_2. Considering the best scenario when the nitrification-denitrification is carried out through nitrite, the amount of consumed oxygen would be of 3.4 kg O_2/kg NH_4^+-$N_{removed}$. In the case of partial nitritation and Anammox, in one or two units, the consumed oxygen is reduced in a 63%, and only 1.9 kg O_2 are necessary for the process. Therefore, the estimated energy consumption in an nitrification-denitrification plant is of 2.4 kWh/kg $N_{removed}$ and in the case of partial nitritation-Anammox it would be 1 kWh/kg $N_{removed}$ (Joss *et al.*, 2009). If it is considered that the use of an anaerobic digester can produce biogas (CH_4 and CO_2) which accounts for around 3.5 kWh/kg $COD_{removed}$, the energy balance of the farm could be even positive.

Having in mind the results already described in Chapter 5 from the aerobic granular sludge technology organic matter removal efficiencies up to 88% and nitrogen removal efficiencies up to 70% were obtained when the applied OLR was of 4.4 kg COD/(m^3 d) and NLR was of 0.81 kg N/(m^3 d) (Figure 6.8). Further increase in loading rate caused a decrease in the treated NLR, so those could be reported as threshold values for this system for the achievement of simultaneous organic matter and nitrogen removal. The use of a CANON reactor as a post-treatment of the effluent generated from the aerobic granular reactor made it possible to reduce the ammonia content in the effluent when the nitrogen removal efficiency in the first unit was limited. In terms of removal efficiency, the obtained results in the CANON system were satisfactory.

When the process for organic matter and nitrogen removal is analyzed the following question arises: Which is the best option: a GSBR treating simultaneously organic matter and nitrogen or a GSBR-CANON treating higher OLR and NLR?.

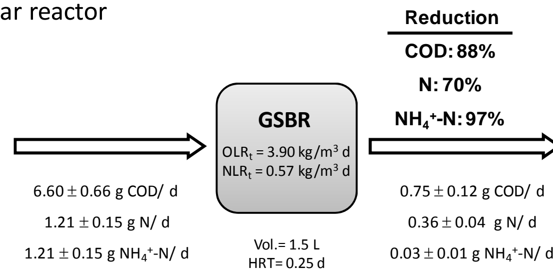
An analysis based on the treated loads was performed in order to compare the results obtained with the GSBR when working with an applied NLR of 0.81 kg N/(m^3 d) with the configuration of a GSBR-CANON working with 1.26 kg N/(m^3 d).

The following considerations were done:

- The daily flow treated in the GSBR was 6 L/d, so one unit of 3 L is needed for the CANON reactor to treat all the generated effluent (HRT of the CANON reactor of 12 h).
- The average values of COD, total and ammonia nitrogen of the GSBR correspond to those obtained during the Stage ID (Chapter 5).

- The $OLR_{treated}$ and the $NLR_{treated}$ in the CANON system were kept in the same value as that obtained in this chapter. A theoretical value of the effluent and the overall removal efficiencies were calculated.

- The $OLR_{treated}$ and the $NLR_{treated}$ by the two units was calculated, considering the influent of the GSBR, the theoretical effluent of the CANON and the volume of the two units.



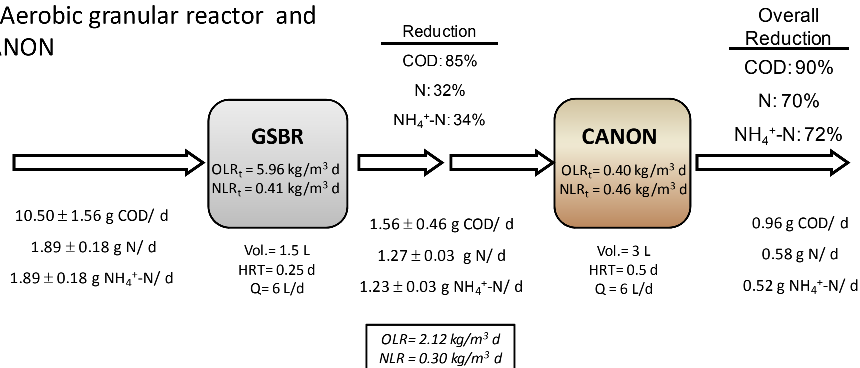


Figure 6.8. COD and N mass balances when working with a single aerobic granular reactor a) and with the combination of two units, an aerobic granular reactor and a CANON system b).

Considering the obtained data from the operation of the GSBR and the CANON reactors the nitrogenous compounds obtained in the effluent would be different in each system. The effluent from the GSBR would be mainly composed of nitrite and nitrate, whereas the effluent of the CANON would be composed of ammonia. With the optimization of operational conditions the composition of nitrogenous compounds in the effluent of both systems would decrease.

Due to the use of two units with a total volume of 4.5 L, the obtained OLR and NLR when working with the two units were of 2.12 and 0.30 kg /(m^3 d). Both loads are lower than those obtained in the GSBR as a single unit. This finding suggest that is better to work with a GSBR treating loading rates that enables the simultaneous nitrification-denitrification than the use of the two units, and in case of treating higher loads is better to increase the volume

of the GSBR. Hence, the proposal of a slurry treatment based in aerobic granular reactor and CANON process could be disadvantageous in terms of costs (higher oxygen requirements and the need of constructing two units) and treated loads.

The use of an aerobic granular reactor would be suitable for those constructed farms with limitations in the space that need to install a treatment process for the generated swine slurry or to replace the anaerobic lagoons. Compared to the conventional nitrification-denitrification process based on activated sludge systems, aerobic granular reactors are designed with a high height to diameter ratio (around 5) and the space requirements are lower. Moreover, the compact structure and good settling properties enable high biomass retention and the possibility to treat high volumetric loads (Liu and Tay, 2004; Campos *et al.*, 2009).

6.5. CONCLUSIONS

The use of a CANON reactor with pulsing air for the nitrogen removal from pre-treated swine slurry was feasible. In spite of the low temperature, the CANON reactor treated a NLR of 0.46 kg N/(m^3 d) and showed good resistance to the variations in the concentration of nitrogen (230 ± 50 mg N/L) and slowly- or non- biodegradable organic matter (260 ± 74 mg COD/L) contained in the pre-treated swine slurry. Most of the organic matter was slowly or non biodegradable. With this setup the ammonia removal efficiency was of 78 %, and the total nitrogen removal efficiency was of 75%.

The biomass present in the reactor was a mixture of anammox enriched granules that coexisted with biomass in suspended form, mainly comprising aerobic ammonia oxidizers. An increase of the mean feret diameter of the granules was observed until reaching a mean feret diameter of 2.6 mm. The development of the biomass in these two phases diminished the competition of ammonia oxidizers, anammox and heterotrophs for common substrates.

The comparison of the aerobic process and other post-treatment for effluents from anaerobic digesters showed that the CANON technology is promising for its application in farms if compounds such as antibiotics, high concentrations of organic matter or coagulants are avoided. The system can operate at moderated temperature (20 °C) and the amount oxygen consumed is low.

6.6. REFERENCES

Abma W.R., Schultz C.E., Mulder J.W., van der Star W.R., Strous M., Tokutomi T. and van Loosdrecht M.C. (2007). Full-scale granular sludge Anammox process. *Water Science and Technology,* 55(8-9), 27-33.

Ahn Y.H., Hwang I.S. and Min K.S. (2004). ANAMMOX and partial denitritation in anaerobic nitrogen removal from piggery waste. *Water Science and Technology,* 49(5-6), 145-153.

An J.Y., Kwon J.C., Ahn D.W., Shin D.H., Shin H.S. and Kim B.W. (2007). Efficient nitrogen removal in a pilot system based on upflow multi-layer bioreactor for treatment of strong nitrogenous swine wastewater. *Process Biochemistry,* 42(5), 764-772.

APHA-AWWA-WPCF (2005) Standard methods for the examination of water and wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Beline F., Daumer M.L., Loyon L., Pourcher A.M., Dabert P., Guiziou F. and Peu P. (2008). The efficiency of biological aerobic treatment of piggery wastewater to control nitrogen, phosphorus, pathogen and gas emissions. *Water Science and Technology,* 57(12), 1909-1914.

Bernet N. and Béline F. (2009). Challenges and innovations on biological treatment of livestock effluents. *Bioresource Technology,* 100(22), 5431-5436.

Burton C.H. (1992). A review of the strategies in the aerobic treatment of pig slurry: Purpose, theory and method. *Journal of Agricultural Engineering Research,* 53, 249-272.

Campos J.L., Figueroa M., Mosquera-Corral A. and Mendez R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering,* 1, 136-151.

Chamchoi N., Nitisoravut S. and Schmidt J.E. (2008). Inactivation of ANAMMOX communities under concurrent operation of anaerobic ammonium oxidation (ANAMMOX) and denitrification. *Bioresource Technology,* 99(9), 3331-3336.

Cheng J. and Liu B. (2001). Nitrification/Denitrification in intermittent aeration process for swine wastewater treatment. *Journal of Environmental Engineering,* 127(8), 705-711.

Choi E. and Eum Y. (2002). Strategy for nitrogen removal from piggery waste. *Water Science and Technology,* 46(6-7), 347-354.

Dapena-Mora A., Fernandez I., Campos J.L., Mosquera-Corral A., Mendez R. and Jetten M.S.M. (2007). Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology,* 40(4), 859-865.

de Graaf A.A.V., deBruijn 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.

Dosta J., Fernández I., Vázquez-Padín J.R., Mosquera-Corral A., Campos J.L., Mata-Álvarez J. and Méndez R. (2008). Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials,* 154(1-3), 688-693.

EPA (2002) Anaerobic Lagoons. Wastewater Technology Fact Sheet. Document EPA 832-F-02-009, Environmental Protection Agency (EPA), Washington DC.

Fernandez I., Mosquera-Corral A., Campos J.L. and Mendez R. (2009). Operation of an Anammox SBR in the presence of two broad-spectrum antibiotics. *Process Biochemistry,* 44(4), 494-498.

Fernández I., Dosta J., Fajardo C., Campos J.L., Mosquera-Corral A. and Méndez R. Short- and long-term effects of ammonium and nitrite on the Anammox process. *Journal of Environmental Management,* In Press, Corrected Proof. doi: 10.1016/j.jenvman.2010.10.044

Figueroa M., Val del Río A., Campos J.L., Mosquera-Corral A. and Mendez R. (2011). Treatment of high loaded swine slurry in an aerobic granular reactor. *Water Science and Technology*.

Flotats X., Bonmatí A., Fernández B. and Magrí A. (2009). Manure treatment technologies: On-farm versus centralized strategies. NE Spain as case study. *Bioresource Technology,* 100(22), 5519-5526.

Furukawa K., Inatomi Y., Qiao S., Quan L., Yamamoto T., Isaka K. and Sumino T. (2009). Innovative treatment system for digester liquor using anammox process. *Bioresource Technology,* 100(22), 5437-5443.

Güven D., Dapena A., Kartal B., Schmid M.C., Maas B., van de 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.

Hao X.D. and van Loosdrecht M.C.M. (2004). Model-based evaluation of COD influence on a partial nitrification-Anammox biofilm (CANON) process. *Water Science and Technology,* 49(11-12), 83-90.

Holm-Nielsen J.B., Al Seadi T. and Oleskowicz-Popiel P. (2009). The future of anaerobic digestion and biogas utilization. *Bioresource Technology,* 100(22), 5478-5484.

Hwang I.S., Min K.S., Choi E. and Yun Z. (2005). Nitrogen removal from piggery waste using the combined SHARON and ANAMMOX process. *Water Science and Technology,* 52(10-11), 487-494.

Hwang I.S., Min K.S., Choi E. and Yun Z. (2006). Resource recovery and nitrogen removal from piggery waste using the combined anaerobic processes. *Water Science and Technology,* 54(8), 229-236.

Isaka K., Sumino T. and Tsuneda S. (2007). High nitrogen removal performance at moderately low temperature utilizing anaerobic ammonium oxidation reactions. *Journal of Bioscience and Bioengineering,* 103(5), 486-490.

Joss A., Salzgeber D., Eugster J., König R., Rottermann K., Burger S., Fabijan P., Leumann S., Mohn J. and Siegrist H. (2009). Full-Scale Nitrogen Removal from Digester Liquid with Partial Nitritation and Anammox in One SBR. *Environmental Science & Technology,* 43(14), 5301-5306.

Karakashev D., Schmidt J.E. and Angelidaki I. (2008). Innovative process scheme for removal of organic matter, phosphorus and nitrogen from pig manure. *Water Research,* 42(15), 4083-4090.

Kartal B., van Niftrik L., Rattray J., van de Vossenberg J.L.C.M., Schmid M.C., Sinninghe Damste J., Jetten M.S.M. and Strous M. (2008). Candidatus "Brocadia fulgida": an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiology Ecology,* 63(1), 46-55.

Kumar M. and Lin J.-G. (2010). Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal--Strategies and issues. *Journal of Hazardous Materials,* 178(1-3), 1-9.

Lackner S., Terada A. and Smets B.F. (2008). Heterotrophic activity compromises autotrophic nitrogen removal in membrane-aerated biofilms: Results of a modeling study. *Water Research,* 42(4-5), 1102-1112.

Liu Y. and Tay J.H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances,* 22(7), 533-563.

Loughrin J.H., Vanotti M.B., Szogi A.A. and Lovanh N. (2009). Evaluation of second-generation multistage wastewater treatment system for the removal of malodors from liquid swine waste. *Journal of Environmental Quality,* 38(4), 1739-1748.

Molinuevo B., García M.C., Karakashev D. and Angelidaki I. (2009). Anammox for ammonia removal from pig manure effluents: Effect of organic matter content on process performance. *Bioresource Technology,* 100(7), 2171-2175.

Møller H.B., Sommer S.G. and Ahring B.K. (2004). Methane productivity of manure, straw and solid fractions of manure. *Biomass and Bioenergy,* 26(5), 485-495.

Mosquera-Corral A., Gonzalez F., Campos J.L. and Mendez R. (2005). Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds. *Process Biochemistry,* 40(9), 3109-3118.

Nielsen M., Bollmann A., Sliekers O., Jetten M., Schmid M., Strous M., Schmidt I., Larsen L.H., Nielsen L.P. and Revsbech N.P. (2005). Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. *FEMS Microbiology Ecology,* 51(2), 247-256.

Park H., Rosenthal A., Ramalingam K., Fillos J. and Chandran K. (2010). Linking community profiles, gene expression and N-removal in Anammox bioreactors treating municipal anaerobic digestion reject water. *Environmental Science & Technology,* 44(16), 6110-6116.

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

Strous M., Kuenen J.G. and Jetten M.S.M. (1999). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology,* 65(7), 3248-3250.

Tijhuis L., van Benthum W.A.J., van Loosdrecht M.C.M. and Heijnen J.J. (1994). Solids retention time in spherical biofilms in a biofilm airlift suspension reactor. *Biotechnology and Bioengineering,* 44(8), 867-879.

Tilche A., Bortone G., Malaspina F., Picinini S. and Stante L. (2001). Biological nutrient removal in a full-scale SBR treating piggery wastewater: results and modelling. *Water Science and Technology,* 43(3), 363-371.

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(4), 397-405.

Vanotti M.B., Szogi A.A., Millner P.D. and Loughrin J.H. (2009). Development of a second-generation environmentally superior technology for treatment of swine manure in the USA. *Bioresource Technology,* 100(22), 5406-5416.

Vázquez-Padín J., Fernádez I., Figueroa M., Mosquera-Corral A., Campos J.-L. and Méndez R. (2009a). Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature. *Bioresource Technology,* 100, 2988-2994.

Vázquez-Padín J.R., Pozo M.J., Jarpa M., Figueroa M., Franco A., Mosquera-Corral A., Campos J.L. and Méndez R. (2009b). Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR. *Journal of Hazardous Materials,* 166(1), 336-341.

Wett B. (2006). Solved upscaling problems for implementing deammonification of rejection water. *Water Science and Technology,* 53(12), 121-128.

Yamamoto T., Takaki K., Koyama T. and Furukawa K. (2008). Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by Anammox. *Bioresource Technology,* 99(14), 6419-6425.

Yang J., Zhang L., Hira D., Fukuzaki Y. and Furukawa K. High-rate nitrogen removal by the Anammox process at ambient temperature. *Bioresource Technology,* In Press, Corrected Proof.

Yoshino M., Yao M., Tsuno H. and Somiya I. (2003). Removal and recovery of phosphate and ammonium as struvite from supernatant in anaerobic digestion. *Water Science and Technology,* 48(1), 171-178.

Chapter 7:

Characterization of the performance and microbial populations from a CANON reactor treating predigested swine slurry

Summary

The nitrogen removal performance and the responsible microbial community for the processes involved were studied in an air pulsing CANON reactor operated at room temperature. The CANON reactor was operated for more than 120 days, as a post-treatment of predigested swine slurry, treating nitrogen loading rates of around 0.46 kg N/(m^3 d) when a concentration of 300 mg N/L was applied to the system.

Inside the reactor the biomass grew in the form of granules (comprising mainly anammox bacteria) with a feret diameter of 2.6 mm that coexisted with dispersed biomass (containing mainly ammonia oxidizing bacteria). In order to characterize these microorganisms molecular techniques, including DGGE/cloning on the basis of the PCR targeting 16S rRNA and the amoA gene, and FISH, were conducted. Results revealed that the dominant anammox bacteria belonged to the species *C.* Brocadia fulgida and that the ammonia oxidizers corresponded to the species *Nitrosomonas europaea*. Other types of bacteria such as *Bacteroidetes*, *Comamonadaceae* and *Zoogloea* with the potential of consuming dissolved oxygen and organic matter under micro-aerobic conditions were also identified. The biomass from the CANON reactor presented a high biodiversity which allowed for the stability of the system and the treatment of relatively high nitrogen loads at room temperature.

Keywords: anammox, AOB, CANON, microbial populations, swine slurry.

7.1. INTRODUCTION

The discovery of the anammox process in the second half of the nineties promoted a deep change in the understanding of the biological nitrogen cycle (Strous and Jetten, 2004). Anammox bacteria were found to contribute significantly to the nitrogen gas production in oceans (Kuypers *et al.*, 2005) and they have been also proposed as an alternative to remove ammonia from high nitrogen loaded wastewater in the absence of organic matter and oxygen on both full and laboratory scale reactors (Kartal *et al.*, 2004; van der Star *et al.*, 2007).

When the anammox process is intended to be applied for the treatment of low C/N containing wastewaters the combination of two processes is required: a) the aerobic process of partial nitritation where 50% of ammonia is oxidized to nitrite and b) the anoxic anammox process where previously produced nitrite and left ammonia are converted to nitrogen gas producing a small amount of nitrate. Partial nitrification and anammox processes can be performed in two different units or simultaneously in a single one in the so called Completely Autotrophic Nitrogen removal Over Nitrite process (CANON) (Sliekers *et al.*, 2002).

Under oxygen-limiting conditions a co-culture of aerobic and anaerobic ammonium oxidizing bacteria are established in the CANON system mainly grown in the form of biofilms or granules (Helmer *et al.*, 2001). In this system, ammonium is partly converted to nitrite by ammonium oxidizing bacteria (AOB) using the low dissolved oxygen concentrations present, and subsequently, anammox bacteria combine ammonium with nitrite to form nitrogen gas. Typically in the CANON process the *Nitrosomonas*-like AOB are active in the outer aerobic region of both biofilm and aggregates, while anammox bacteria are active in the inner anoxic region. In this way, the anammox bacteria are protected from dissolved oxygen, which is consumed in the outer aerobic region (Nielsen *et al.*, 2005; Vázquez-Padín *et al.*, 2010).

Since the discovery of anammox bacteria, several different representatives have been enriched from biomass collected in WWTPs. These are the *Candidatus* lineages *Kuenenia* with "K. stuttgartiensis"(Schmid *et al.*, 2000), *Brocadia* with the species "B. anammoxidans" (Strous *et al.*, 1999) and "B. fulgida" (Kartal *et al.*, 2008) and *Anammoxoglobus* with "A. propionicus" (Kartal *et al.*, 2007). One important fact is that a successful enrichment of some anammox bacteria was achieved using a medium containing propionate ("A. propionicus") or acetate ("B. fulgida") and these were found to be able to co-oxidize organic compounds such as formate, propionate, monomethylamine and dimethylamine at high rates and in this way out-compete the other anammox species for nitrite.

Regarding the anammox application bacterial diversity in CANON systems treating industrial wastewater is expected to be significant and influenced by the occurrence of variable environmental physical and chemical conditions. For example, having in mind that microorganisms such as nitrite/nitrate denitrifying bacteria have been found in systems treating effluents free of organic matter (Kindaichi et al., 2007; Liang et al., 2009; Zheng et al.,

2005), even higher shares of these microorganisms are expected in a CANON reactor treating wastewater with low organic matter contents. Although a competition for nitrite as electron acceptor between the denitrifying bacteria and the anammox communities is expected (Tang *et al.*, 2010), it has been shown that anammox bacteria can, in some cases, outcompete successfully the heterotrophic denitrifiers (Güven *et al.*, 2005; Kartal *et al.*, 2007).

The availability of molecular tools, such as those used to analyze the 16S ribosomal DNA, have made possible to explore the microbial diversity in complex environments (Daims et al., 2006b; Rittmann, 2006) and provide information which enables to understand the performance of the reactor systems.

7.2. OBJECTIVE

The aim of this study is to identify the AOB, anammox and heterotrophic bacteria in a CANON reactor used as a post-treatment for nitrogen removal of swine slurry. The microbial community structure was researched by a molecular approach, including qualitative fluorescence *in situ* hybridization (FISH) as well as polymerase chain reaction (PCR) amplification targeting the 16S rRNA and *amoA* genes, denaturing gradient gel electrophoresis (DGGE) and cloning.

7.3. MATERIALS AND METHODS

7.3.1. Reactor description and operational conditions

The description of the pulsing sequencing batch reactor (SBRP) and the applied operational conditions are extensible described in Chapter 6. The day zero of operation was fixed in this work to correspond to the date since when the pre-treated effluent was fed to the system. The effluent pre-treatment was performed in an aerobic granular reactor where the oxidation of the organic matter occurred.

7.3.2. Sample collection, DNA extraction and PCR amplification

Biomass samples were collected from the CANON reactor on operating days -9 (prior to the change of the feeding), 29, 66 and 113. Biomass was harvested directly from the SBR and sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher). DNA was extracted using the MoBio Power Soil™ kit (MoBio Laboratories) according to the manufacturer's protocol. DNA was then suspended in 50 µL of ultrapure water and kept at 4 °C until further analysis.

The extracted DNA was PCR amplified using an automated thermal cycler (Applied Biosystems). The primers used in this study and the PCR conditions are shown in Table 7.1. The reactions were conducted in a final volume of 25 µL, using GoTaq Green Master Mix

(Promega) and 1 μL of template DNA. The PCR products were checked by electrophoresis in 1% agarose gel and fragment sizes were estimated using a DNA ladder (Fermentas).

Table 7.1. PCR primers and experimental conditions used in this study.

Primer	Sequence	Target	Ref.	PCR conditions
PLA46F	GACTTGCATGCCTAATCC	Planctomycetes	[1]	5 min at 96 °C, followed by 35 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.
630R	CAKAAAGGAGGTGATCC			
amoA 1F	GGGGTTTCTACTGGTGGT	AOB	[2]	5 min at 96 °C, followed by 35 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.
amoA 2R	TCCCCCTCTGCAAAGCCTTCTTC			
341F*	CCTACGGGAGGCAGCAG	16s RNA gene	[3]	9 min at 95 °C, followed by 25 cycles of 60 s at 94 °C, 60 s at 55 °C, 90 s at 72 °C and 10 min of final extension at 72 °C.
907R	CCGTCAATTCCTTTGAGTTT			
616F	AGAGTTTGATYMTGGCTCAG	16s RNA gene	[4]	5 min at 96 °C, followed by 35 cycles of 45 s at 96 °C, 45 s at 56 °C, 60 s at 72 °C and 5 min of final extension at 72 °C.
630R	CAKAAAGGAGGTGATCC			

[1] Schmid *et al.*, 2003; [2] Nicolaisen and Ramsing, 2002; [3] Yu and Morrison, 2004; [4] Juretschko *et al.*, 1998.
*GC Clamp added for DGGE-PCR: 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG- 3'.

7.3.3. Denaturing Gradient Gel Electrophoresis

The amplified 16S rRNA gene sequences generated with the primers 341F and 907R were separated by DGGE using an Ingeny phorU system (Ingeny). The polyacrylamide gel (6% [wt/vol]) contained a linear formamide/urea gradient ranging from 40% to 75% overlaid with a nondenaturing stacking gel. The gel was run at 100 V for 15 h at 60 °C. The gel was stained with SYBR-Green solution (Invitrogen). After visualization under UV transillumination specific gel bands were excised with a sterilized scalpel and dissolved in 30 μL Milli-Q water, stored at 4 °C overnight and reamplified by PCR using primers 341F-907R.

7.3.4. Cloning and sequencing of 16S rRNA gene and phylogenetic analysis

PCR products were ligated and transformed into *Escherichia coli* using the pGEM-T Easy vector system kit (Promega) according to the manufacturer's instructions. Plasmid DNA was isolated with the Flex-prep kit (Amersham Pharmacia Biotech Inc.) and digested with 5U EcoRI enzyme in EcoRI buffer for 2 h at 37 °C. The digestion products were examined for an insert with the expected size by electrophoresis in 1% agarose gel. Sequencing was done with BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems). The reaction mixtures

were analyzed with the 3700 DNA analyzer (Applied Biosystems). The sequences of the 16S rRNA gene fragments were determined by using the M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site. The sequences of the AmoA gene were determined by using only the M13 forward primer (Suzuki *et al.*, 2001).

Forward and reverse sequences of the 16S rRNA gene were manually inspected using the Contig Express software from the Vector NTI Suite 6.0 (Informax) and analyzed against GenBank by using BLAST search of nucleic acid databases (Altschul *et al.*, 1990). Overlapping fragments of the 16S rRNA gene of *Planctomycetes* were assembled into a single contiguous sequence using Contig Express software (Invitrogen). Sequences were aligned with ClustalW and phylogenetic trees were constructed in MEGA4 (Tamura *et al.*, 2007) using the neighbor-joining method and bootstrap test with 100 replications.

7.3.5. Fluorescent *in situ* hybridization

Bacterial populations were identified by the Fluorescence *in situ* hybridization (FISH) technique. Biomass samples from the reactor were collected, disrupted and fixed according to the procedure described by Amann *et al.*, 1995. Details of oligonucleotide probes are available at probeBase (Loy *et al.*, 2007). Used probes and percentages of formamide are shown in Table 7.2. Fluorescence signals were recorded with an acquisition system Coolsnap (Roper Sicientific Photometrics) coupled to an Axioskop 2 epifluorescence microscope (Zeiss). A confocal laser scanning microscope (CLSM) (TCS-SP2, Leica) was also used.

Table 7.2. Targeted organisms and the corresponding formamide percentages (%F) for the used oligonucleotide probes.

Probe	Probe sequence (5'→3')	%F	Targeted organisms
PLA46	GACTTGCATGCCTAATCC	30	Planctomycetales
BFU613	GGATGCCGTTCTTCCGTTAAGCGG	30	*Candidatus* "Brocadia fulgida"
BAN162	CGGTAGCCCCAATTGCTT	40	*Candidatus* "Brocadia sp"
NSO190	CGATCCCCTGCTTTTCTCC	55	Betaproteobacterial ammonia-oxidizing bacteria
NEU653*	CCCCTCTGCTGCACTCTA	40*	Most of the halophilic and halotolerant *Nitrosomonas* spp.
CF319a	TGGTCCGTGTCTCAGTAC	35	most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*
CF319b	TGGTCCGTATCTCAGTAC	35	some *Flavobacteria* and *Sphingobacteria*
CFB562	TACGYWCCCTTTAAACCCA	30	subgroup of *Bacteroidetes*
CTE	TTCCATCCCCCTCTGCCG	20	*Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp., *Aquaspirillum* spp.
ZRA23	CTGCCGTACTCTAGTTAT	35	Most members of the *Zoogloea* lineage, not *Z. resiniphila*

* Used with an equimolar amount of corresponding unlabeled competitor oligonucleotide probe.

Quantification of microbial populations, based on a biovolume fraction, was performed after the application of the FISH technique with the *Daime* software (Daims et al., 2006a).

7.4. RESULTS AND DISCUSSION

7.4.1. Reactor performance

Experimental results obtained in the CANON reactor, in terms of nitrogen and organic matter removal, were presented in Chapter 6. However, in order to permit a rapid visualization of the experimental data and the operational conditions of each biomass sampling day, the following figures (Figure 7.1) are included. Biomass samples during the operation of the reactor fed with pre-digested effluent were collected on days 29, 66 and 113. A sample was previously collected on day -9 (before the feeding change) to be used as a reference one.

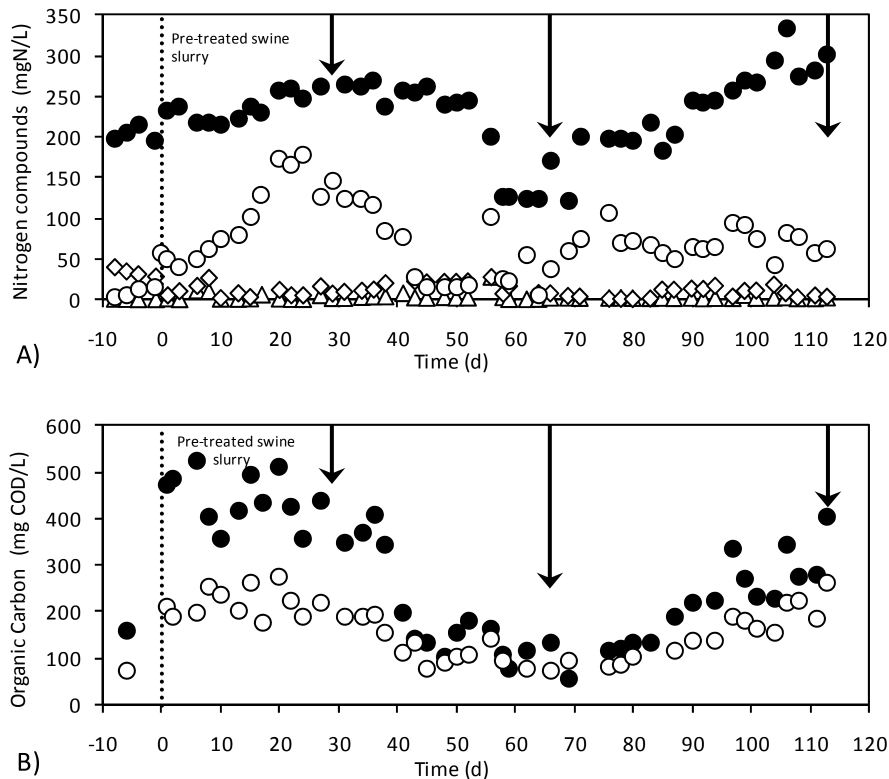


Figure 7.1. A) Evolution of the nitrogen compounds concentrations (mg N/L) in the CANON reactor: NH_4^+ in the influent (●) and NH_4^+ (○), NO_2^- (△) and NO_3^- (◇) in the effluent. B) Evolution of organic matter concentration (mg COD/L) in the CANON reactor: COD in the influent (●) and in the effluent (○). Each arrow (➔) corresponds to a day of biomass sampling.

7.4.2. Biomass physical properties

In this CANON system, the biomass was developed in two different states: granules mainly composed of anammox bacteria (identified by their reddish colour), and biomass in suspension, mainly composed of AOB and heterotrophic bacteria (Figure 7.2).

Figure 7.2. Images of the reactor and the biomass inside the reactor on day 58 of operation.

The fact that mainly AOB grew as dispersed biomass could be a strategy of these microorgannisms to overcome substrate diffusion limitation due to the low DO concentrations in the bulk liquid and to outcompete heterotrophs (Mosquera-Corral et al., 2005). Moreover, the growth of anammox bacteria mainly in granules and nitrifiers and heterotrophs mainly in suspension presents several advantages for all of them:

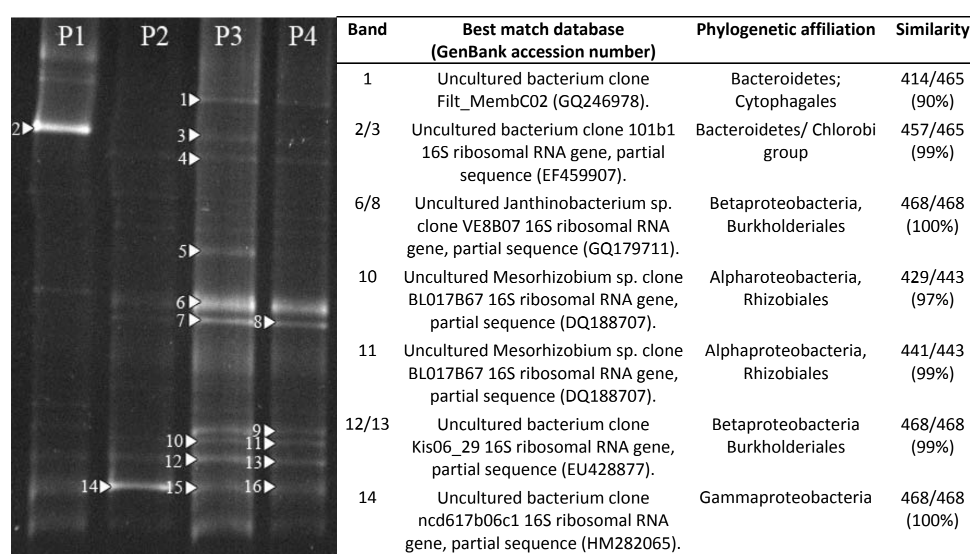
1. The anammox bacteria are protected from the presence of dissolved oxygen, that causes inhibition, since the major part of the oxygen is consumed by the biomass in suspension and by a thin layer of nitrifiers that cover the surface of the granules (Nielsen et al., 2005). It must be pointed out that the DO concentration in the liquid media can be kept in the air pulsing SBR in lower levels than in the conventional CANON process where both populations grow in the granules.

2. Heterotrophs and nitrifiers have easy access to substrates in the liquid media.

3. The anammox inside the granules are not diluted with heterotrophs. Excessive mixed growth of anammox, nitrifiers and heterotrophs inside the granules can lead to a competition for the space which is lost by the anammox, especially because of the large differences of biomass yields between these populations (van Benthum et al., 1997).

7.4.3. DGGE analysis

The DGGE patterns for the selected samples in the different operational days are shown in Figure 7.3. Comparison of DGGE profiles showed that bands were visible at approximately 40-55% of denaturant concentration and that fingerprints of the microbial community slightly changed during the operational period. To further research of the bacterial community, several intense bands were sequenced and the results were aligned with previously published sequences from the NCBI database (Figure 7.3) and the phylogenetic tree was built as indicated and presented in Figure 7.4.



Band	Best match database (GenBank accession number)	Phylogenetic affiliation	Similarity
1	Uncultured bacterium clone Filt_MembC02 (GQ246978).	Bacteroidetes; Cytophagales	414/465 (90%)
2/3	Uncultured bacterium clone 101b1 16S ribosomal RNA gene, partial sequence (EF459907).	Bacteroidetes/ Chlorobi group	457/465 (99%)
6/8	Uncultured Janthinobacterium sp. clone VE8B07 16S ribosomal RNA gene, partial sequence (GQ179711).	Betaproteobacteria, Burkholderiales	468/468 (100%)
10	Uncultured Mesorhizobium sp. clone BL017B67 16S ribosomal RNA gene, partial sequence (DQ188707).	Alpharoteobacteria, Rhizobiales	429/443 (97%)
11	Uncultured Mesorhizobium sp. clone BL017B67 16S ribosomal RNA gene, partial sequence (DQ188707).	Alphaproteobacteria, Rhizobiales	441/443 (99%)
12/13	Uncultured bacterium clone Kis06_29 16S ribosomal RNA gene, partial sequence (EU428877).	Betaproteobacteria Burkholderiales	468/468 (99%)
14	Uncultured bacterium clone ncd617b06c1 16S ribosomal RNA gene, partial sequence (HM282065).	Gammaproteobacteria	468/468 (100%)

Figure 7.3. DGGE profiles of 40%-70% denaturant gradient. Lane P1: sample of day -9; lane P2: sample of day 29; lane P3: sample of day 66; lane P4: sample of day 113 and nucleotide sequence identity of the successfully analyzed DGGE fragments. Bands that had the same best match are grouped together.

Sequences matched with bacterial species from different phyla. Bands 10 and 11 matched with the order of *Rhizobiales* in the class of α-Proteobacteria. Band 14 belonged to the class Gammaproteobacteria and matched with sequences of the order *Chromatiales*. Bands 6 and 8 are closely related to *Janthinobacterium*, and bands 12 and 13 to the family of *Acidovorax* bands, all of them in the order *Burkholderiales* in the class of Betaproteobacteria. Band 1 was related to the order *Cytophagales* and Band 2 and 3 were related to phylum *Chlorobi*, grouped together into the formerly known as *Cytophaga-Flexibacter-Bacteroides* (CFB) division.

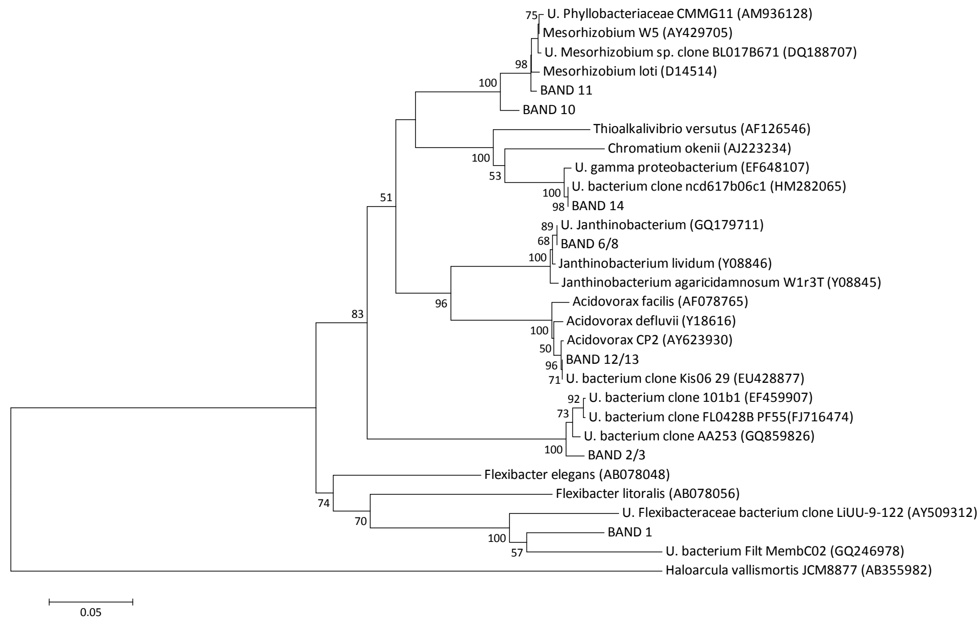


Figure 7.4. Phylogenetic tree constructed by the neighbor-joining method based on DGGE partial 16S rRNA gene sequences (about 460 bp) from the CANON reactor. Bootstrap values (>50) based on 100 replicates are indicated. The evolutionary distances were computed using the Maximum Composite Likelihood method. *Haloarcula vallismortis* was used as outgroup.

7.4.4. Phylogenetic analysis of anammox and AOB by cloning

None of the sequences retrieved from the DGGE were related to AOB or anammox bacteria, but this result did not fit with the macroscopic results obtained from the reactor operation. In order to avoid this phenomenon of underrepresentation of the 16S rRNA gene sequences (Schmid *et al.*, 2005) a cloning approach was applied to sample P2 (day 29). This sample corresponded to an operational period when 0.26 kg N/(m^3 d) were removed by the CANON system.

The 16S rRNA gene sequences of *Planctomycetes* were obtained after amplification using PCR with the primers Pla46F and 630R and cloning. Twenty nine clones were randomly picked for sequencing, and by applying forward and reverse primers a full-length 16S rRNA gene sequence was generated with a size of 1551 bp. This sequence branched within the *Candidatus* Brocadia lineage and matched 100% with *Candidatus* “Brocadia fulgida” (Figure 7.5).

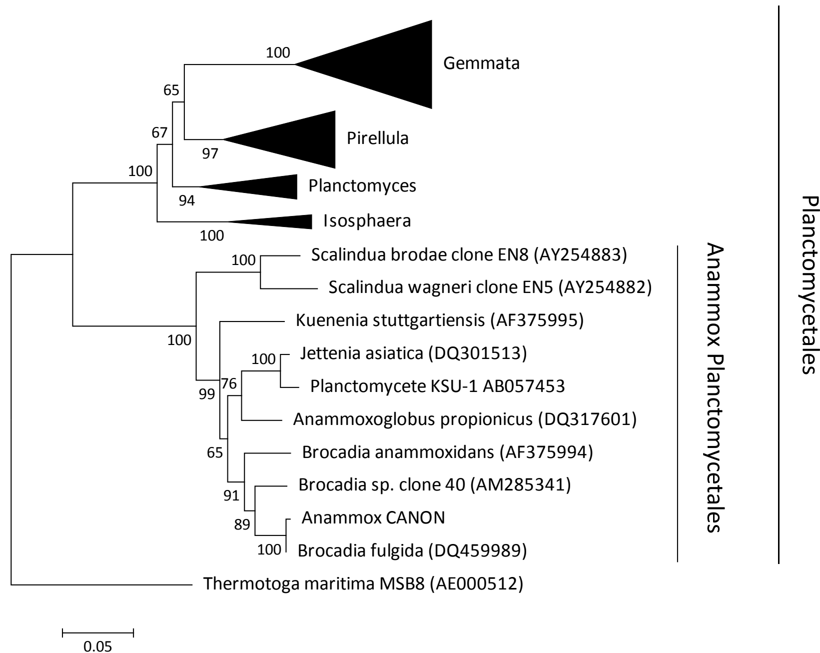


Figure 7.5. Phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences of Planctomycetales. Bootstrap values (>50) based on 100 replicates are indicated. The evolutionary distances were computed using the Maximum Composite Likelihood method. Thermotoga maritima was used as outgroup.

The identification of *Candidatus* "Brocadia fulgida" in an anammox reactor treating wastewater is not frequent. Therefore in bioreactors for wastewater treatment in full-scale or pilot plant scale, the populations usually detected are closely related to *Kuenenia* species or *C.* "Brocadia anammoxidans" (van der Star *et al.*, 2007).

The presence of anammox bacteria closely related to *C.* "Brocadia fulgida" has been recently reported in a reactor treating reject water from an anaerobic digester of municipal sludge (Park *et al.*, 2010). The presence of organic matter or nitrite in this CANON reactor could enhance the development of *C*. "Brocadia fulgida" due to the fact that continuous cultivation experiments showed that in the presence of acetate, ammonium, nitrite and nitrate, this specie out-competed other anammox bacteria (Kartal *et al.*, 2008).

The use of amoA gene as a molecular marker for ammonia oxidizers showed that the sample harbored two different Nitrosomonads (*Nitrosomonas europaea* and Uncultured *Nitrosomonadaceae* bacterium) (Figure 7.6). The presence of *Nitrosomonas* was expected due to the imposed operational conditions in the reactor with ammonium concentrations in excess (Schramm *et al.*, 1998). Previous studies of identification of microbial populations in CANON reactors reported *Nitrosomonas* as the main population involved in the ammonia aerobic oxidation (Sliekers *et al.*, 2002; Vázquez-Padín *et al.*, 2010; Vlaeminck *et al.*, 2010).

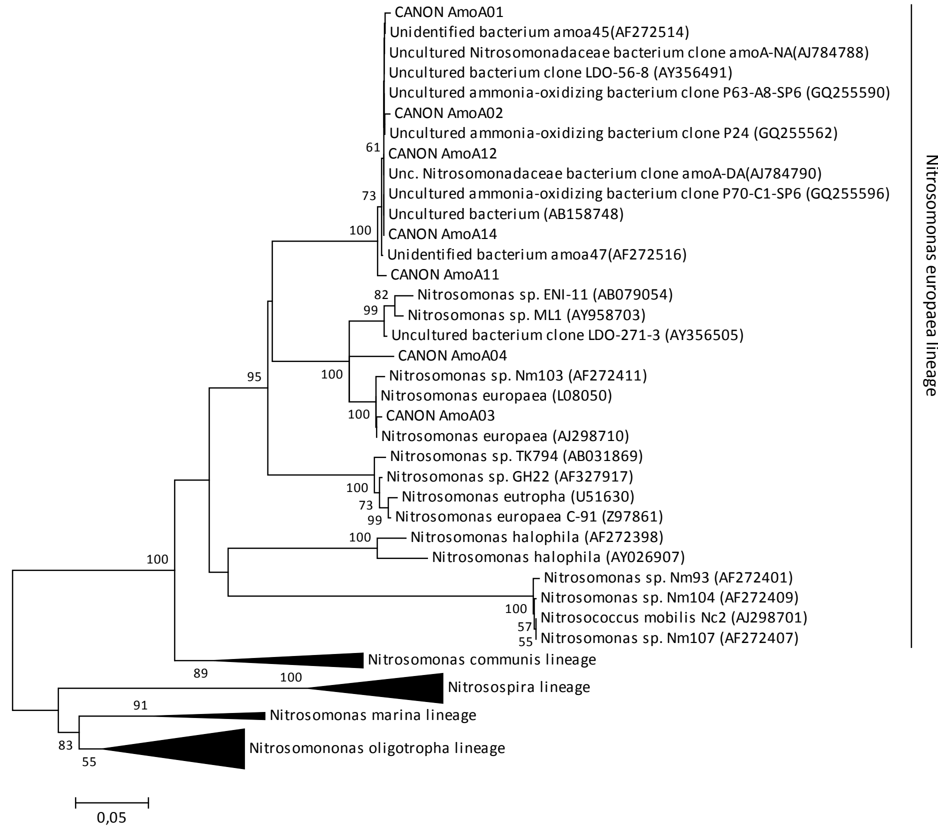


Figure 7.6. Phylogenetic tree constructed by the neighbor-joining method of the AmoA gene sequences retrieved from the CANON system. The evolutionary distances were computed using the Maximum Composite Likelihood method and evolutionary analyses were conducted in MEGA4 (Tamura et al., 2007).

7.4.5. FISH analysis

In order to visualize the distribution of the main bacteria populations present in the reactor and detected by the previous applied methodologies, the FISH technique was applied to a biomass sample collected on day 29.

Probes NSO190 and NEU653 showed that bacteria belonging to the genus *Nitrosomonas* were widely present and they were found as a dominant population in the sample. Referring to the biovolume fraction, these bacteria accounted for around the 35% of the total biomass and formed spherical clusters. They also appeared as individual cells in the surroundings of the anammox bacteria.

The use of probes PLA46 and BFU613 confirmed that *C*. "Brocadia fulgida" was the dominant anammox bacteria present in the sample, with an estimated relative percentage of 25% of the population (Figure 7.7A). One interesting feature of this anammox bacterium was the presence of autofluorescent extracellular substances, observed for the first time by Kartal et al., (2008) in anammox bacteria. This autofluorescence hindered the use of FITC labelled probes for the visualization of these bacteria under the epifluorescence microscope. Although all the sequences retrieved from the clone library were closely related to *C*. "Brocadia fulgida" the other Brocadia specie (*C.* "Brocadia anammoxidans") was also detected by FISH. The abundance of this specie is estimated in a 5%. Although different genera of anammox bacteria are seldom found in the same habitat, in this case, both Brocadia species coexisted in the same reactor. Comparison with one sample collected on day -9 showed that both species were present in the reactor before the change of the feeding, although the abundance of *C*. "Brocadia fulgida" was similar to *C.* "Brocadia anammoxidans".

From the retrieved sequences of DGGE (Figure 7.3) the existence of heterotrophic bacteria belonging to the phylum *Bacteroidetes* was observed. These organisms are known to play an important role in the turnover of organic matter (Cottrell and Kirchman, 2000) and were also observed in swine faeces and manure storage pits (Cotta *et al.*, 2003). The presence of bacteria belonging to the phylum *Chlorobi* and *Bacteroidetes* was also reported in anammox reactors by the use of clone libraries (Li *et al.*, 2009) but the role of these bacteria in such environment is not clear (Cho *et al.*, 2010). The application of CF319a/b and CFB562 revealed a high abundance of *Bacteroidetes*. These rod-shape bacteria were distributed in dispersed form accounting for around 10% of the total biomass (Figure 7.7B).

From the application of probe CTE a 5% of bacteria belonging to the *Comamonadaceae* family was observed distributed in dispersed form among the rest of biomass (Figure 7.7). Bacteria hybridized with probe CTE could correspond with bacteria of the genus *Acidovorax* as observed by the sequences of DGGE gel (Figure 7.3).

A large number of *Comamonadaceae* and also bacteria belonging to *Bacteroidetes* were observed by FISH in a biomass sample collected from the aerobic granular reactor (data not shown) where the treated effluent was produced. Their presence in the CANON reactor would suggest that these heterotrophic bacteria moved from the aerobic granular reactor with the effluent to the CANON one.

Members of the family *Rhodocyclaceae* were also observed after the application of probe ZRA23a, belonging to the genus *Zoogloea*, and accounted for less than 1% of the total population. Studies performed in WWTP treating industrial wastewater revealed that several betaproteobacterial genera belonging to the families *Comamonadaceae* and *Rhodocyclaceae* are usually present as dominating denitrifiers (Thomsen *et al.*, 2007).

The role of the heterotrophic bacteria in this reactor could be related to the removal of rests of slowly biodegradable organic matter. The coexisting nitrifying and heterotrophic bacteria consume the oxygen or organic by-products of anammox bacteria, which consequently prevented inhibition by dissolved oxygen and accumulation of organic waste products. However, an excessive growth of the coexisting bacteria may negatively affect the activity of the system.

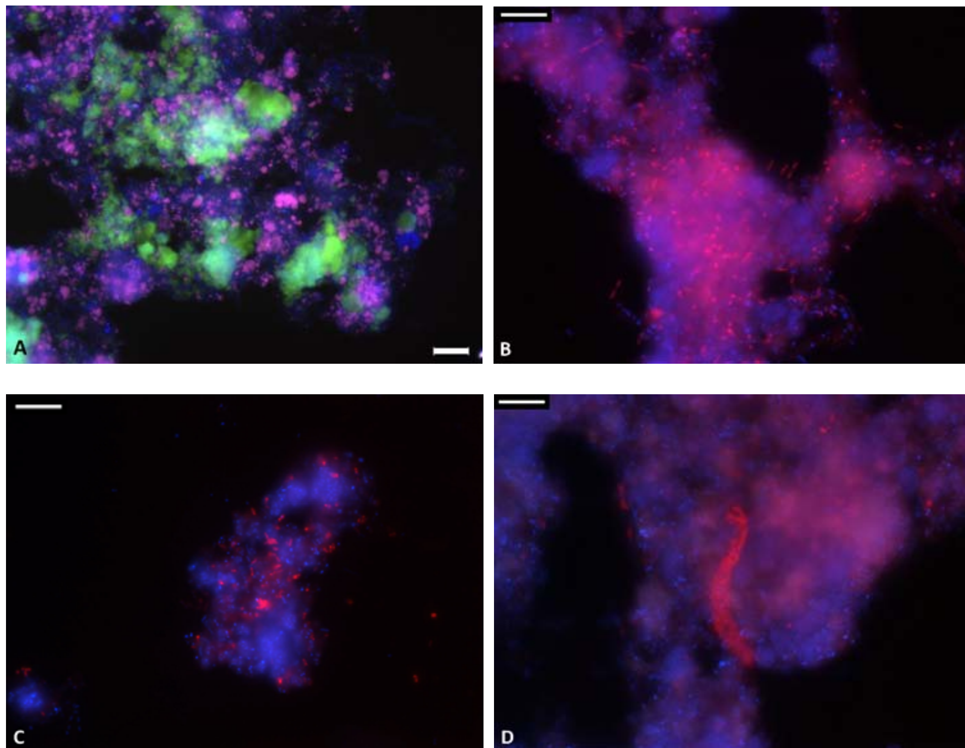


Figure 7.7. FISH analysis images of the biomass sample collected on day 29. A) CLSM image where AOB appear in pink (hybridized with Cy3-labelled NEU653 probe), *C.* Brocadia appear in green (hybridized with FITC-labelled BAN162) and other Bacteria appear in blue (hybridized with Cy5-labelled EUBMIX probe). Bar = 100 µm. B) Bacteroidetes appear in pink hybridized with Cy3-labelled CF319a/b probe) and the rest in blue (DAPI). C) Comamonadacea bacteria appear in pink (hybridized with Cy3-labelled Cte probe) and the rest in blue (DAPI). D) Zoogloea bacteria appear in pink (hybridized with Cy3-labelled ZRA23a probe) and the rest in blue (DAPI). D). The bar indicates 10 µm.

7.5. CONCLUSIONS

The coexistence of anammox and AOB activities was achieved in a CANON reactor treating swine slurry at room temperature. The use of air pulses favoured the development of granular and dispersed biomass comprising mainly anammox bacteria and AOB, respectively.

The microorganisms playing a key role in the nitrogen removal process were identified. *C*. "Brocadia fulgida", *C.* "Brocadia anammoxidans" formed the anammox biomass, accounting around of 30% of total biomass. *Nitrosomonas* bacteria were identified as the ammonia oxidizers responsible of the CANON process, in a percentage around 35%. The coexistence between AOB and anammox was well-supported by the experimental results of the reactor performance and those obtained with the application of the FISH technique.

The presence of slow- or non-biodegradable organic matter promoted the development of heterotrophic bacteria but it did not cause that heterotrophs rapidly outcompeted anammox bacteria. *Bacteroidetes*, *Comamonadacea*e and *Rhodocyclaceae* would be the major contributors to organic removal when treating swine slurry.

7.6. REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Amann, R., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiological Reviews *59*, 143-169.

Cotta, M.A., Whitehead, T.R., and Zeltwanger, R.L. (2003). Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. Environmental microbiology *5*, 737-745.

Cottrell, M.T., and Kirchman, D.L. (2000). Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. *66*, 5116-5122.

Cho, S., Takahashi, Y., Fujii, N., Yamada, Y., Satoh, H., and Okabe, S. (2010). Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. Chemosphere *78*, 1129-1135.

Daims, H., Lucker, S., and Wagner, M. (2006a). Daime, a novel image analysis program for microbial ecology and biofilm research. Environmental Microbiology *8*, 200-213.

Daims, H., Taylor, M.W., and Wagner, M. (2006b). Wastewater treatment: a model system for microbial ecology. Trends Biotechnol. *24*, 483-489.

Güven, D., Dapena, A., Kartal, B., Schmid, M.C., Maas, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., Op den Camp, H.J.M., Jetten, M.S.M.*, et al.* (2005). Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria. Appl. Environ. Microbiol. *71*, 1066-1071.

Helmer, C., Tromm, C., Hippen, A., Rosenwinkel, K.H., Seyfried, C.F., and Kunst, S. (2001). Single stage biological nitrogen removal by nitritation and anaerobic ammonium oxidation in biofilm systems. Water Sci. Technol. *43* 311-320.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P., and Wagner, M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. Appl. Environ. Microbiol. *64*, 3042-3051.

Kartal, B., Rattray, J., van Niftrik, L.A., van de Vossenberg, J., Schmid, M.C., Webb, R.I., Schouten, S., Fuerst, J.A., Damste, J.S., Jetten, M.S.M., and Strous, M. (2007). Candidatus "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. *30*, 39-49.

Kartal, B., van Niftrik, L., Rattray, J., van de Vossenberg, J.L.C.M., Schmid, M.C., Sinninghe Damste, J., Jetten, M.S.M., and Strous, M. (2008). Candidatus "Brocadia fulgida": an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol. Ecol. *63*, 46-55.

Kartal, B., van Niftrik, L., Sliekers, O., Schmid, M.C., Schmidt, I., van de Pas-Schoonen, K., Cirpus, I., van der Star, W., van Loosdrecht, M., Abma, W.R.*, et al.* (2004). Application, eco-physiology and biodiversity of anaerobic ammonium-oxidizing bacteria. Reviews in Environmental Science and Biotechnology *3*, 255-264.

Kindaichi, T., Tsushima, I., Ogasawara, Y., Shimokawa, M., Ozaki, N., Satoh, H., and Okabe, S. (2007). In situ activity and spatial organization of anaerobic ammonium-oxidizing (Anammox) bacteria in biofilms. Appl. Environ. Microbiol. *73*, 4931-4939.

Kuypers, M.M.M., Lavik, G., Woebken, D., Schmid, M., Fuchs, B.M., Amann, R., Jorgensen, B.B., and Jetten, M.S.M. (2005). Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proc. Natl. Acad. Sci. U. S. A. *102*, 6478-6483.

Li, X.-R., Du, B., Fu, H.-X., Wang, R.-F., Shi, J.-H., Wang, Y., Jetten, M.S.M., and Quan, Z.-X. (2009). The bacterial diversity in an anaerobic ammonium-oxidizing (anammox) reactor community. Syst. Appl. Microbiol. *32*, 278-289.

Liang, Z., Liu, J.-X., and Li, J. (2009). Decomposition and mineralization of aquatic humic substances (AHS) in treating landfill leachate using the Anammox process. Chemosphere *74*, 1315-1320.

Loy, A., Maixner, F., Wagner, M., and Horn, M. (2007). probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic Acids Res. *35*, D800-D804.

Mosquera-Corral, A., Gonzalez, F., Campos, J.L., and Mendez, R. (2005). Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds. Process Biochem. *40*, 3109-3118.

Nicolaisen, M.H., and Ramsing, N.B. (2002). Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J. Microbiol. Methods *50*, 189-203.

Nielsen, M., Bollmann, A., Sliekers, O., Jetten, M., Schmid, M., Strous, M., Schmidt, I., Larsen, L.H., Nielsen, L.P., and Revsbech, N.P. (2005). Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. FEMS Microbiol. Ecol. *51*, 247-256.

Park, H., Rosenthal, A., Ramalingam, K., Fillos, J., and Chandran, K. (2010). Linking community profiles, gene expression and N-removal in Anammox bioreactors treating municipal anaerobic digestion reject water. Environ. Sci. Technol. *44*, 6110-6116.

Rittmann, B.E. (2006). Microbial ecology to manage processes in environmental biotechnology. Trends Biotechnol. *24*, 261-266.

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

Schmid, M., Walsh, K., Webb, R., Rijpstra, W.I.C., van de Pas-Schoonen, K., Verbruggen, M.J., Hill, T., Moffett, B., Fuerst, J., Schouten, S., *et al.* (2003). Candidatus "Scalindua brodae", sp nov., Candidatus "Scalindua wagneri", sp nov., two new species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. *26*, 529-538.

Schmid, M.C., Maas, B., Dapena, A., Pas-Schoonen, K.V., de Vossenberg, J.V., Kartal, B., van Niftrik, L., Schmidt, I., Cirpus, I., Kuenen, J.G., *et al.* (2005). Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. Appl. Environ. Microbiol. *71*, 1677-1684.

Schramm, A., de Beer, D., Wagner, M., and Amann, R. (1998). Identification and activities in situ of Nitrosospira and Nitrospira spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. *64*, 3480-3485.

Sliekers, A.O., Derwort, N., Campos, J.L., Strous, M., Kuenen, J.G., and Jetten, M.S.M. (2002). Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res. *36*, 2475-2482.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., and Jetten, M.S.M. (1999). Missing lithotroph identified as new planctomycete. Nature *400*, 446-449.

Strous, M., and Jetten, M.S.M. (2004). Anaerobic oxidation of methane and ammonium. Annu. Rev. Microbiol. *58*, 99-117.

Suzuki, Y., Yoda, T., Ruhul, A., and Sugiura, W. (2001). Molecular cloning and characterization of the gene coding for Azoreductase from Bacillus sp. OY1-2 isolated from soil. J. Biol. Chem. *276*, 9059-9065.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. *24*, 1596-1599.

Tang, C.-j., Zheng, P., Wang, C.-h., and Mahmood, Q. (2010). Suppression of anaerobic ammonium oxidizers under high organic content in high-rate Anammox UASB reactor. Bioresour. Technol. *101*, 1762-1768.

Thomsen, T.R., Kong, Y., and Nielsen, P.H. (2007). Ecophysiology of abundant denitrifying bacteria in activated sludge. FEMS Microbiol. Ecol. *60*, 370-382.

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. Biotechnol. Bioeng. *53*, 397-405.

van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.W., Tokutomi, T., Strous, M., Picioreanu, C., and van Loosdrecht, M.C.M. (2007). Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. Water Res. *41*, 4149-4163.

Vázquez-Padín, J., Mosquera-Corral, A., Campos, J.L., Méndez, R., and Revsbech, N.P. (2010). Microbial community distribution and activity dynamics of granular biomass in a CANON reactor. Water Res. *44*, 4359-4370.

Vlaeminck, S.E., Terada, A., Smets, B.F., De Clippeleir, H., Schaubroeck, T., Bolca, S., Demeestere, L., Mast, J., Boon, N., Carballa, M., and Verstraete, W. (2010). Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox. Appl. Environ. Microbiol. *76*, 900-909.

Yu, Z., and Morrison, M. (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-Denaturing Gradient Gel Electrophoresis. Appl. Environ. Microbiol. *70*, 4800-4806.

Zheng, X.-s., Yang, H., and Li, D.-t. (2005). Change of microbial populations in a suspended-sludge reactor performing completely autotrophic N-removal. World Journal of Microbiology and Biotechnology *21*, 843-850.

Chapter 8:

Presence of filamentous bacteria in different aerobic granular reactors

Summary

Aerobic granules are dense and compact biofilms that are mainly composed by bacteria and also contain extracellular polymeric substances (EPS), protozoa and, in some cases, fungi. Special members of the bacterial community in wastewater treatment processes are the filamentous bacteria because of their implication in bulking and foaming processes, in activated sludge systems, and settling problems, in biofilms. The use of the fluorescence *in situ* hybridization (FISH) technique allowed the visualization of filamentous bacteria in three different aerobic granular reactors treating industrial wastewater. *Thiothrix* and *Chloroflexi* bacteria were observed in an aerobic granular reactor treating wastewater from a fish canning industry, *Meganema perideroedes* was observed in a reactor treating wastewater from a plant processing marine products and *Chloroflexi* and *Sphaerotilus natans* were observed in a reactor fed with synthetic wastewater supplemented with coagulant and flocculant.

Keywords: aerobic granulation, *Chloroflexi,* filamentous bacteria, FISH, *Meganema perideroedes, Sphaerotilus natans, Thiothrix*

8.1. INTRODUCTION

Aerobic granulation is a microbial self-immobilization process that is driven by diverse selection pressures such as the organic loading rate, the hydrodynamic shear force, the presence of a feast-famine regime, the feeding strategy, the reactor configuration, the cycle time or the exchange ratio (Qin *et al.*, 2004; Campos *et al.*, 2009). It is a gradual process, involving progression from suspended sludge to aggregates and further to aerobic granules with a regular outer shape and compact structure (Tay *et al.*, 2001).

Aerobic granules are usually presented as spherical compact aggregates of microorganisms, mainly bacteria, and extracellular polymeric substances (EPS), and under some certain conditions with filamentous outgrowth (Tay *et al.*, 2001; McSwain *et al.*, 2004; Mosquera-Corral *et al.*, 2005; Liu and Liu, 2006; Figueroa *et al.*, 2008). It has been stated that low-levels of filamentous growth do not cause operational problems and may even stabilize the granule structure (Liu and Liu, 2006). If filamentous growth is dominant in the reactor the settleability of the aerobic granules becomes poor and subsequent biomass washout and eventual disappearance of aerobic granules can occur. The possible causes of filamentous growth identified in activated sludge systems can also be applied to aerobic granular reactors. These factors include long solids retention times, low substrate concentrations in the liquid phase, a high substrate gradient within the granule, dissolved oxygen deficiency, temperature shifts and flow patterns (Liu and Liu, 2006). Because of the cyclic operation of aerobic granular sludge sequencing batch reactors (SBR) and the peculiarities of aerobic granules, various stressing forces can be present simultaneously and can result in the progressive development of filamentous growth.

Filamentous bacteria are normally present in the activated sludge plants in small amounts and under some conditions they proliferate to such an extent that they markedly affect the treatment plant performance. They are often responsible for bulking (inadequate separation of solids and liquid effluent phases) or foaming (solids transported to the surface of either process tank or clarifier) in many activated sludge wastewater treatment plants worldwide. In order to control the growth of these problematic bacteria, their identification was necessary and therefore, more than 30 different filamentous bacterial types have been described on the basis of their morphology (Eikelboom, 2000; Jenkins *et al.*, 2003).

Nowadays, the identification based on morphotypes is known to be frequently inadequate for the reliable identification of many filamentous bacteria, since one morphotype can embrace several phylogenetically very different organisms. For example, studies have revealed that the *Nostocoida limicola* morphotypes are affiliated with members of the class *Alphaproteobacteria* (Snaidr *et al.*, 2002), the phyla *Chloroflexi* (Schade *et al.*, 2002), *Firmicutes* (Liu *et al.*, 2000) and *Actinobacteria* (Liu and Seviour, 2001) and the order *Planctomycetales* (Liu *et al.*, 2001).

The application of molecular techniques has begun to resolve the taxonomic status of some of these bacteria (Martins *et al.*, 2004; Nielsen *et al.*, 2009b), as shown in Table 8.1. Furthermore the use of molecular methods, especially FISH with rRNA-directed oligonucleotides, has been particularly productive in their identification. For example, *Sphaerotilus natans* and *Leptothrix discophora* are difficult to identify because they share an almost identical morphology, but with the design of specific probes (Wagner et al., 1994) their differentiation nowadays is much easier. The combination of FISH with microautoradiography (Lee *et al.*, 1999) also expanded the existent knowledge about their ecophysiology, information that might provide clues to know the ways to avoid their appearance in the reactor and to control the problems they cause (Nielsen *et al.*, 2009b).

Table 8.1. Overview of the phylogenetic affiliation, species name (if possible) and corresponding morphotype (adapted from Nielsen *et al.*, 2009a).

Phylogenetic identity Phylum/class	**Genus/Species name**	**Morphotype**
Proteobacteria/ Alphaproteobacteria	*Candidatus* Alysiosphaera europea	*Nostocoida limicola*
	Candidatus Monilibacter batavus	*N. limicola*
	Candidatus Alysiomicrobium bavaricum	*N. limicola*
	Candidatus Combothrix itálica	*N. limicola*
	Meganema perideroedes	Type 021N
Proteobacteria/ Betaproteobacteria	Genus *Curvibacter*	Type 1701
	Genus *Curvibacter*	Type 0041/0675
	Sphaerotilus natans	*Sphaerotilus natans*
	Leptothrix discophora	*Leptothrix*
Proteobacteria/ Gammaproteobacteria	*Thiothrix nivea, T. unzii, T. fructosivorans, T. defluvii*	*Thiothrix* species
	T. eikelboomii, T. disciformis, T. flexilis	Type 021 N species
	Acinetobacter spp.	Type 1863
	Leucothrix mucor	*Leucothrix mucor*
	Beggiatoa spp.	*Beggiatoa*
Bacteroidetes	*Haliscomenobacter hydrossis*	*H. hydrossis*
	Other *H. hydrossis-like* species	*H. hydrossis*-like
	Unknown species	Type 0092
	Chryseobacterium spp.	Type 1863
Chloroflexi	*Kouleothrix aurantiaca*	Type 1851
	Unknown species	*N. limicola* II
	Other *Chloroflexi,* (species not identified)	Thin *H. hydrossis*-like (- epiphytic growth)
	Other *Chloroflexi,* (species not identified)	Type 1851 (+ epiphytic growth)
	Other *Chloroflexi,* (species not identified)	Type 0041/0675 (± epiphytic growth)

8.2. OBJECTIVE

The main objective of this work was to compile the observations made using the FISH technique about the presence of filamentous bacteria in aerobic granules collected from three different SBR reactors treating different wastewater. In order to understand the reasons for the development of these bacteria in the aerobic granules, the operational conditions of these systems and the main physical characteristics of the granules at the time of the sample collection were analyzed.

8.3. MATERIALS AND METHODS

8.3.1. Experimental set-up

Three sequencing batch reactors (SBRs) with a working volume of 1.5 L were used. The dimensions of the units were: height of 465 mm and inner diameter of 85 mm, therefore the height to the diameter ratio (H/D) was of 5.5. Experimental set-up was the same in the three cases. Oxygen was supplied by using air spargers to promote the formation of small bubbles to guarantee the complete mixture and the oxygen transfer. Two peristaltic pumps were used to introduce the feeding solution, on top of the reactor, and to discharge the effluent, at medium height in the column reactor, respectively. The liquid exchange volume was fixed at 50%. The actuations of the pumps and valves and the different periods of the operational cycle were controlled by a programmable logic controller (S7-224CPU, Siemens).

The SBRs were operated in cycles of 3 hours and distributed as follows: 3 minutes of feeding, 171 minutes of aeration, 1 minute of settling and 5 minutes of effluent withdrawal.

8.3.2. Operational conditions

Two of the reactors were fed with industrial wastewater. The first one (R1) was fed with the effluent produced in a fish canning industry with high and low salinity. The second one (R2) was fed with the wastewater from a plant processing marine products. The third reactor (R3) was fed with a synthetic medium, containing sodium acetate as carbon source according to Beun *et al.* (1999), supplemented with a residual quantity of coagulant (2.5 mg/L of polychloride of aluminium) and flocculant (1.5 mg/L of polyelectrolyte). A fourth reactor (R3b) was run in parallel under the same operational conditions as R3, but without coagulant and flocculant, considered only to compare the bacterial population with those detected in R3.

8.3.3. Fluorescence *in situ* hybridization

Samples of granular biomass were collected from the reactors on different operating days for the application of the FISH technique. Analyzed samples were collected from R1 on days 268 and 344, from R2 on days 192 and 263 and from R3 on days 84 and 115. A second

set of samples corresponding to day 99 for R1, day 94 for R2 and the inoculum for R3 were also analyzed and used as references of presence or absence of filamentous bacteria.

To achieve the granular biomass breakage, samples were sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher). The FISH technique was performed according to the procedure described in Chapter 2. Used probes are listed in Table 8.2 and details are available at probeBase (Loy *et al.*, 2007). The oligonucleotide probes were labelled with the fluorochromes Cy3 and FITC. After *in situ* hybridization slides were embedded with Vectashield H-1200 containing DAPI. Fluorescence signals were recorded with an acquisition system coupled to an epifluorescence microscope (Axioskop 2, Zeiss).

Table 8.2. List of 16S and 23S rRNA targeted oligonucleotide probes used for *in situ* identification of filamentous bacteria, formamide percentages (F%) and target organisms.

Probe	Target site	Probe sequence (5'→3')	% F	Target organisms	Ref[a]
EUB338I[b]	338-355	GCTGCCTCCCGTAGGAGT	0-50	Bacteria domain	[1]
EUB338II[b]	338-355	GCAGCCACCCGTAGGTGT	0-50	Planctomycetales	[2]
EUB338III[b]	338-355	GCTGCCACCCGTAGGTGT	0-50	Verrucomicrobiales	[2]
ALF1B	19-35	CGTTCGYTCTGAGCCAG	20	Alphaproteobacteria	[3]
NOLI644	644-660	TCCGGTCTCCAGCCACA	35	*Candidatus* Alysiosphaera europaea	[4]
PPX3	1428-1445	TGGCCCACCGGCTTCGGG	50	*Candidatus* Alysiomicrobium bavaricum	[4]
DF198	198-217	ATCCCAGGGCAACATAGTCT	35	*Candidatus* Monilibacter batavus	[5]
COMBO1031	1031-1050	CACCTGCAGTGGCCTCCCGA	35	*Candidatus* Combothrix italica	[6]
MEG983[b]	983-1001	CGGGATGTCAAAAGGTGG	35	*Meganema perideroedes*	[7]
MEG1028[b]	1028-1046	CTGTCACCGAGTCCCTTGC	35	*Meganema perideroedes*	[7]
BET42a*	1027-1043	GCCTTCCCACTTCGTTT	35	*Betaproteobacteria*	[3]
Competitor		GCCTTCCCACATCGTTT			
CURVI997*	997-1014	CTCTGGTAACTTCCGTAC	35	*Curvibacter delicatum* ATCC14667; *Pseudomonas lanceolata* AB021390 and few more Betaproteobacteria	[8]
Competitor1		CTCTGGCAACTTCCGTAC			
Competitor2		CTCTGGTCACTTCCGTAC			
LDI	649-666	CTCTGCCGCACTCCAGCT	35	Leptothrix discophora	[9]
SNA	656-673	CATCCCCCTCTACCGTAC	45	Sphaerotilus natans	[10]
GAM42a*	1027-1043	GCCTTCCCACATCGTTT	35	Gammaproteobacteria	[3]
Competitor		GCCTTCCCACTTCGTTT			
G123T*	697-714	CCTTCCGATCTCTATGCA	40	*Thiothrix eikelboomii, T. nivea, T. unzii, T. fructosivorans, T. Defluvii*	[11]
Competitor		CCTTCCGATCTCTACGCA			
CFX1223	1223-1242	CCATTGTAGCGTGTGTGTMG	35	phylum *Chloroflexi*	[12]
GNSB941	941-957	AAACCACACGCTCCGCT	35	phylum *Chloroflexi*	[13]
EUK516	502-51	ACCAGACTTGCCCTCC	25	Eukarya	[1]
MY1574	1474-1489	TCCTCGTTGAAGAGC	20	Eumycota (Fungi)	[14]

[a][1] Amann *et al.*, 1990; [2] Daims *et al.*, 1999; [3] Manz *et al.*, 1992; [4] Snaidr *et al.*, 2002; [5] Nittami *et al.*, 2009; [6] Levantesi *et al.*, 2004; [7] Thomsen *et al.*, 2006; [8] Thomsen *et al.*, 2004; [9] Wagner *et al.*, 1994; [10]

Wagner *et al.*, 1994; [11] Kanagawa *et al.*, 2000; [12] Bjornsson *et al.*, 2002; [13] Gich *et al.*, 2001; [14] Baschien, 2003.
[b]$EUB338_{mix}$ probe is an equimolar mixture of probes EUB338I, EUB338II and EUB338III, and MEG_{mix} of MEG983 and MEG1028.
*Probes that were used in a 1:1 ratio together with their specific probe competitors.

8.3.4. Analytical methods

The pH, conductivity, ammonia (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), volatile suspended solids (VSS), total suspended solids (TSS) concentrations and the sludge volumetric index (SVI) were determined according to the Standard Methods (APHA-AWWA-WPCF, 2005) and the methods reported in Chapter 2. Chemical Oxygen Demand (COD) was determined by a semi-micro method (Soto *et al.*, 1989). Biomass density was determined using dextran blue (Beun *et al.*, 1999).

The size distribution and morphology of the granules were determined regularly by using an image analysis procedure (Tijhuis *et al.*, 1994) with a stereomicroscope (Stemi 2000-C, Zeiss) and by scanning electron microscopy (SEM) (Digital SEM 440, Leica). For SEM analysis the sludge samples were washed with phosphate buffer and 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 %).

8.4. RESULTS AND DISCUSSION

The sludge used as inoculum in all the reactors was a fresh flocculent activated sludge from a wastewater treatment plant located in Calo (Milladoiro) collected in different dates. In all cases during the first days of operation an almost complete washout of the suspended biomass was observed as a result of the operational strategy applied to the systems. Afterwards, the process of granules formation started, in a similar way to that proposed by Beun *et al.*, (1999). Although the time needed to obtain stable granules was different in each system, according to the special features of each feeding composition.

Detailed information about the operation of these systems is provided in Chapter 3 of this thesis and in Val del Río *et al.* (*accepted*).

8.4.1. R1: Effluent from a fish canning industry

Operational conditions

This reactor was operated during 400 days in those operational conditions which consisted of a stepwise increase of organic matter and nitrogen concentrations (Figure 8.1). During the different operational stages nitrogen removal efficiencies and concentrations of nitrogen oxides experienced variations. From day 149 to 376 the NaCl content in the feeding was reduced from 10 g NaCl/L to 0.1 g NaCl/L. The performance of the system from day 268 to 344 is characterized by the increase of the organic (OLR) and nitrogen loading rates (NLR).

This increase involved a change from 540 mg COD/L to 840 mg COD/L of organic matter in the influent. On day 344 the system was fed with an OLR around 3.4 kg COD_s/m^3 d and a NLR of 0.37 kg N/m^3 d. Organic matter removal efficiencies were of 91% and 95% on days 268 and 344, respectively while nitrogen removal efficiencies were of 31% and 26%, respectively, achieved mainly via denitrification.

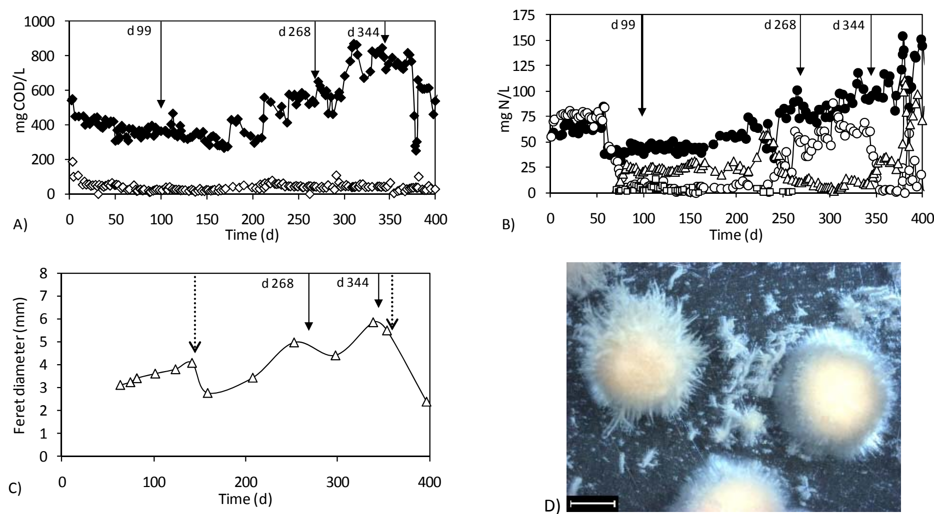


Figure 8.1. Performance of R1 and sampling dates (days 268 and 344) indicated with arrows. A) Organic matter concentration (mg COD/L): COD_{inf} (◆), COD_{eff} (◇) and B) nitrogen compounds concentrations (mg N/L): NH_4^+-N_{inf} (●), NH_4^+-N_{eff} (○), NO_2^--N_{eff} (△). C) Evolution of the feret diameter (mm) and breakage episodes indicated with dotted arrows. D) Picture of the granules on day 339 with the bar indicating 2 mm.

Due to the aforementioned operational conditions of the reactor, (Chapter 3) the formation of the aerobic granules was delayed and did not take place in the “traditional” way (Beun *et al.*, 1999) with the formation of small pellets. At the beginning of the operational period, a phase where elongated structures were formed instead of granules occurred due to dissolved oxygen limitation. Only after the breakage of these initial structures the aggregates were developed. They were characterized by a fluffy aspect with small fibrous structures on their surface that gradually disappeared. Stable granules were observed after day 75, with an average diameter of 2.2 mm. From day 268 to 344 the feret diameter of the granules increased from 4.8 to 6 mm on day 355. At this point the breakage of the biggest granules in small ones was observed. During the whole operational period filamentous outgrowth on the surface of the granules was observed by means of a stereomicroscope.

FISH observations of filamentous bacteria

Filamentous bacteria were observed on the samples collected on days 268 and 344 after the application of $EUB338_{mix}$ probe. General probes for *Alpha*-, *Beta*- and

Gammaproteobacteria were tested, and positive results were only obtained with the probe Gam42a. According to the existent data that related the phylogenetic affiliation of filamentous *Gammaproteobacteria* to their corresponding morphotype (shown in brackets), the organisms possibly present in the sample were: *Thiothrix nivea, T. unzuii, T. fructosivorans, T. defluvii* (Thiothrix species)*, T. eikelboomii, T. disciformis, T. flexilis* (Type 021N species)*, Acinetobacter* spp. (Type 1863)*, Leucotrix mucor (*Leucotrix mucor*)* or *Beggiatoa* spp. (Beggiatoa) (Table 8.1).

In this case, the filamentous *Gammproteobacteria* bacteria present in the sample were identified to belong to genus *Thiothrix* after the identification by DGGE (Chapter 4) and the application of probe G123T (Figure 8.2A, B, C). Filamentous bacteria that belonged to phylum *Chloroflexi* were also identified, and positive hybridizations were obtained after the use of probes CFX1223 (Figure 8.2D) and GNSB941. The length of *Chloroflexi* bacteria was around 30 µm, shorter than the observed length of the *Thiothrix* filamentous, that ranged from 75 to 300 µm (Figure 8.2A), and their abundance was also much lower (Figure 8.2D).

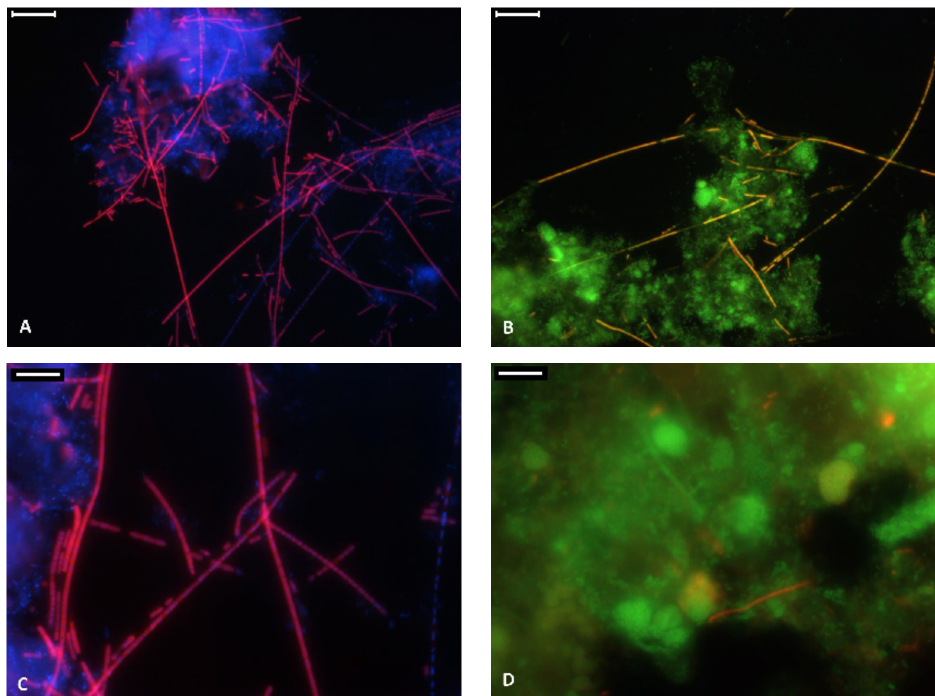


Figure 8.2. FISH images of filamentous bacteria from R1 on day 344. A) Gam42a (Cy3) and DAPI, B) $EUB338_{mix}$ (FITC) and G123T (Cy3), C) DAPI and G123T (Cy3), and D) CFX1223 (Cy3) and $EUB338_{mix}$ (FITC), the bar indicates 25 µm in A) and B) and 10 µm in C) and D).

Thiothrix is a filamentous sulfur bacteria belonging to the class *Gammaproteobacteria* that was found to cause settleability problems in activated sludge processes and loss of solids

from secondary clarifiers (Williams and Unz, 1985). These organisms were reported in microscopy-based surveys of different municipal and industrial wastewater treatment plants (WWTPs) with and without nutrient removal and their occurrence has been found to be favored by influents rich in reduced sulphur compounds or with nutrients deficiency or in systems operated with low feeding to microorganisms (F/M) ratios (Eikelboom, 2000; Nielsen *et al.*, 2000). These bacteria are capable to store sulphur granules and Poly-β-hidroxyalkanoates (PHA); and have rapid nutrients uptake rates under nutrient deficiency. The outgrowth of these bacteria in aerobic granules cultivated with brewery wastewater has been previously reported (Weber *et al.*, 2007) and they were thought to be responsible of the bulking and settling problems, probably caused by the high applied OLR (3.6 kg COD_s/m^3 d), the dissolved oxygen deficiency or the high temperatures of operation reported in that research.

Filamentous members of the phylum *Chloroflexi* have occasionally been associated with bulking incidences in activated sludge systems, but no particular industrial waste was found to favor their presence. They were detected in wastewater treatment systems from different industries as well as in municipal wastewater treatment plants with activated sludge systems (Bjornsson *et al.*, 2002; Kragelund *et al.*, 2007) and also in autotrophic nitrifying granules (Kindaichi *et al.*, 2004; Matsumoto *et al.*, 2010). Although their presence is more relevant in anaerobic methanogenic sludge, where they are also responsible of bulking episodes (Yamada *et al.*, 2005).

The sample used as reference, collected on day 99 of operation, indicated the absence of both filamentous bacteria at that operational stage of the reactor, whereas on day 268 some short filaments of *Thiothrix* and only some members of phylum *Chloroflexi* were detected in the sample. Therefore, it can be concluded that the filamentous bacteria could be present in the industrial wastewater and that the applied operational conditions in the reactor could have favored their further development, like in the case of *Thiothrix* bacteria.

Images obtained with the SEM technique on day 374 (Figure 3.5 from Chapter 3 and Figure 8.3) showed the filamentous microorganisms tightly wrapped and connected with the rest of bacteria and also growing on the surface of the granules. In this case, filamentous bacteria could act as a structural backbone of the aerobic granules.

Even though filamentous bacteria were observed by the FISH technique on days 268 and 344, apparently they did not cause operational problems such as bulking or bad setteability, reflected in a low SVI, during the different operational periods of R1. However, the excessive growth of *Thiothrix* could be responsible of the breakage of the granules observed after day 355. In the case of *Chloroflexi,* due to the low abundance of these filamentous bacteria in the analyzed samples, it can be stated that their presence in this reactor was not relevant for the breakage event of the granular biomass.

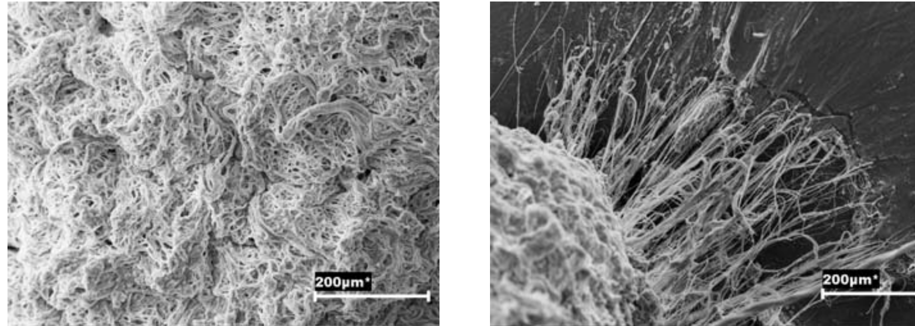


Figure 8.3. SEM images of a granule from R1 sampled on day 374 of operation.

8.4.2. R2: Effluent from a plant processing marine products

Operational conditions of this reactor are shown in Figure 8.4. High COD removal efficiencies were obtained during the major part of the operational time, except for the period between days 98 and 108; however, nitrogen removal efficiency was scarce. Due to the high proteins content of the wastewater and their hydrolysis, the ammonia concentration was even higher in the effluent than in the influent in certain periods. Nitrite, below 25 mg NO_2^--N, was measured only in short periods of time (from days 140 to 190 and 250 to 290) whereas nitrate was not detected.

The operation of this system was characterized by a high variability in the composition of the treated wastewater. From days 192 to 263, the applied OLR ranged between 2.4 and 4.4 kg COD_s/m^3 d, and according to that, the nitrogen composition presented also a high variability meaning that the NLR ranged between 0.2 to 0.5 kg N/m^3 d. Organic matter and nitrogen removal efficiencies on day 192 were of 93 and 12%, and on day 263 were of 93 and 14%, respectively.

Aggregates with a large diameter were quickly developed in this reactor. During the whole operational time the growth in size of the existent granules was observed instead of the formation of new aggregates. On day 35 the average diameter of the aggregates was of 2.97 mm and on day 91 it was of 5.41 mm. This excessive growth of the aggregates provoked that they fell apart and this involved that on day 110 the granules had a mean feret diameter of 3.2 mm. The size of these granules remained between 3.2 and 2.1 mm until day 180. Again, with the increase of the OLR on day 180 a rapid increase of the solids concentration in the reactor was observed with an excessive growth of the granules size that reached a mean feret diameter of 11 mm on day 230 and broke up on day 246. A new granulation event occurred from the remnants of granules and these aggregates presented, on day 280, an average diameter of 2.36 mm, a SVI of 49 mL/g TSS and a density of 39 g $VSS/L_{granule}$.

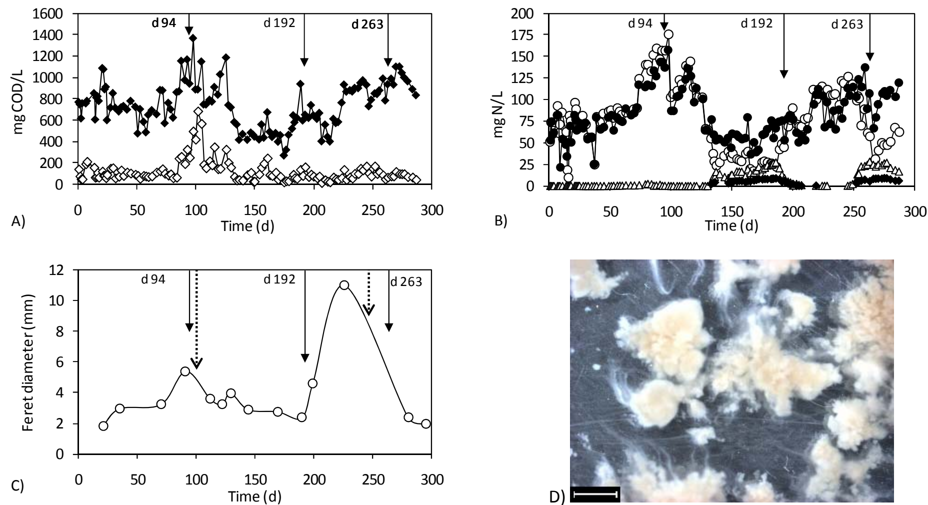


Figure 8.4. Performance of R2 and sampling dates (days 192 and 263) indicated with arrows. A) Organic matter concentration (mg COD/L): COD_{inf} (◆), COD_{eff} (◇); B) nitrogen compounds concentrations (mg N/L): NH_4^+-N_{inf} (●),NH_4^+-N_{eff} (○), NO_2^--N_{eff} (△) in the reactor. C) Evolution of the feret diameter (mm) and breakage episodes indicated with dotted arrows. D) Picture of the granules on day 263 with the bar indicating 2 mm.

FISH observations of filamentous bacteria

When the visualization of the samples collected on days 192 and 263 from R2 was performed, two different types of filaments were observed in both cases.

The biggest filaments resembled fungi hyphae (Figure 8.5A), and this was corroborated by the positive signals obtained once probes EUK516 and MY1574 were applied. Several studies on fungi and protozoa in activated sludge systems demonstrated that these eukaryotic organisms fulfill a wide variety of important tasks in wastewater systems, related to sludge dewaterability and toxic resistance, COD removal, nitrification, or denitrification (Nicolau *et al.*, 2001; Weber *et al.*, 2009). Recent studies showed that an implementation of fungi in aerobic granules usually occurs and that fungal hyphae also act like skeletal elements in the structural formation process (Beun *et al.*, 1999; Weber *et al.*, 2007; Weber *et al.*, 2009).

The other type of observed filaments were curved bacteria that presented positive fluorescent signal with probe Alf1b (Figure 8.5B). According to the phylogenetic affiliations, the genus of filamentous bacteria that belong to class *Alphaproteobacteria* are *Candidatus* Alysiosphaera europaea, *C.* Monilibacter batavus, *C.* Sphaeronema italicum, *C.* Alysiomicrobium bavaricum, *C.* Combothrix italica and *Meganema perideroedes* and all of them can be classified according to a *Nostocoida limicola* or type 021N morphotype (Table 8.1).

From the application of the probes available for the identification of *Alphaproteobacterial* filamentous species in reactor R2 (i.e. NOLI644, PPX3, DF198, COMBO1031, MEG983 and MEG1028, from Table 8.2), positive results were obtained only after the use of probes MEG983 and MEG1028 for *Meganema perideroedes* (Figure 8.5C). By the use of FISH technique it was possible to observe the septa that form the structure of these type of filamentous bacteria, and also their high abundance in the sample (Figure 8.5D).

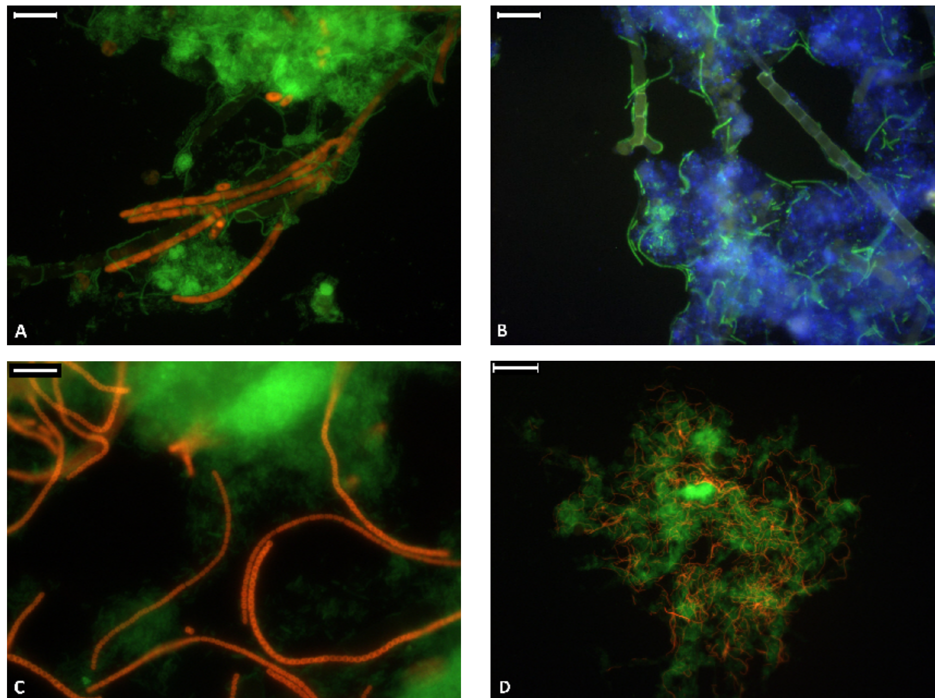


Figure 8.5. FISH images of filamentous organisms from R2 on day 263. A) EUK516 (Cy3) and $EUB338_{mix}$ (FITC) B) Alf1b (FITC) and DAPI. The bar indicates 25 µm in both. C) and D) MEG_{mix} (Cy3) and $EUB338_{mix}$ (FITC). The bar indicates 10 µm and 100 µm, respectively.

FISH-based surveys demonstrated that filamentous *Alphaproteobacteria* were abundant and often involved in bulking and possibly foaming episodes in activated sludge processes. They were present in about 65% of 86 industrial WWTP plants investigated in former studies (van der Waarde *et al.*, 2002; Levantesi *et al.*, 2004). Physiological characteristics of *M. perideroedes* were reported by Kragelund *et al.*, (2005) and among other characteristics, these bacteria are obligate aerobic, oxidase and catalase-positive, consume acetate and glucose under aerobic conditions and seem to have an unusually high substrate uptake capability. The cells contain PHA inclusions and have an extremely high substrate uptake rate and storage capacity, which is probably comparable to or higher than most floc-forming bacteria.

The distribution of the filamentous *Alphaproteobacteria* among different activated sludge plants would suggest that none of them was preferentially associated with a plant treating a particular industrial waste. *Meganema peridoedes* was observed in a completely mixed activated sludge system that received wastewater from a pharmaceutical plant in Grindsted (Denmark) (Kragelund *et al.*, 2005) and it was also detected in two industrial treatment plants, one of them which received wastewater from a tannery and the other from a potato industry (Levantesi *et al.*, 2004). Up to date, the occurrence of *Meganema* in plants treating domestic wastwewater is unknown (Seviour *et al.*, 2010).

When the reference sample taken on day 94 was analyzed, only few fungal hyphae could be observed and *Meganema* bacteria were not detected. However, in the sample taken on day 192, *Meganema* bacteria could be observed, but in lower percentage compared with the amounts observed in the sample collected on day 263 (Figure 8.6A), whereas the amount of eukaryotic cells was higher than that previously observed and similar to sample from day 263 (Figure 8.6B).

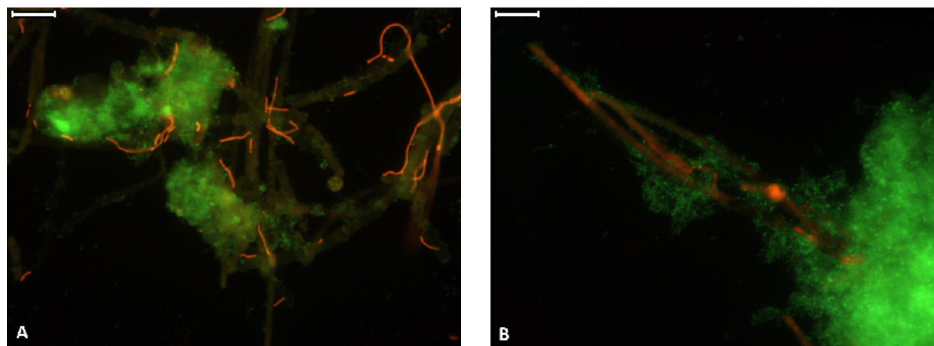


Figure 8.6. FISH images of filamentous organisms from R2 on day 192. A) MEG_{mix} (Cy3) and $EUB338_{mix}$ (FITC). B) EUK516 (Cy3) and $EUB338_{mix}$ (FITC). The bar indicates 25 µm in both.

Therefore, the presence of fungi after 262 days of operation could be explained by their presence in the inoculum or in the raw wastewater, and in the case of *Meganema* by the presence of these microorganisms in the incoming wastewater. From the observations made with the FISH technique in the three different days, the excessive growth of *Meganema* in the granules is clear, and could be related to the increase of the feret diameter of the granules and their further breakage.

8.4.3. R3: Synthetic medium with coagulant-flocculant addition

Operational conditions of the reactor are shown in Figure 8.7. This system is characterized by a non variable organic and nitrogen applied loading rates.

The presence of an easily degradable source of organic matter made it possible to achieve high removal efficiencies during the whole operational time. Nitrogen removal was performed mainly via nitrification-denitrification after 35 days from the start up. In this system nitrite was present during the operation of the reactor in concentrations below 20 mg NO_2^--N/L. By the time of the samples collection the system was fed with an OLR of 2.6 kg COD_s/m^3 d and a NLR of 0.32 kg N/m^3 d. Organic matter removal efficiency on days 84 and 115 was of 94% and 95% and nitrogen removal efficiencies were of 60% and 59%, respectively. Ammonia was removed from the system mainly via nitrification-denitrification (Figure 8.7A,B).

Although small aggregates were observed on day 10 of operation the SVI was of only 100 mL/g TSS. The fast formation of these aggregates with such a high SVI could be related to the presence of the coagulant and floculant contained in the feeding media that promoted the aggregation of the biomass but hindered its compactness. During the whole operational time, filamentous structures were observed on the surface of the granules (Figure 8.7D), and the SVI was over 80 mL/g TSS.

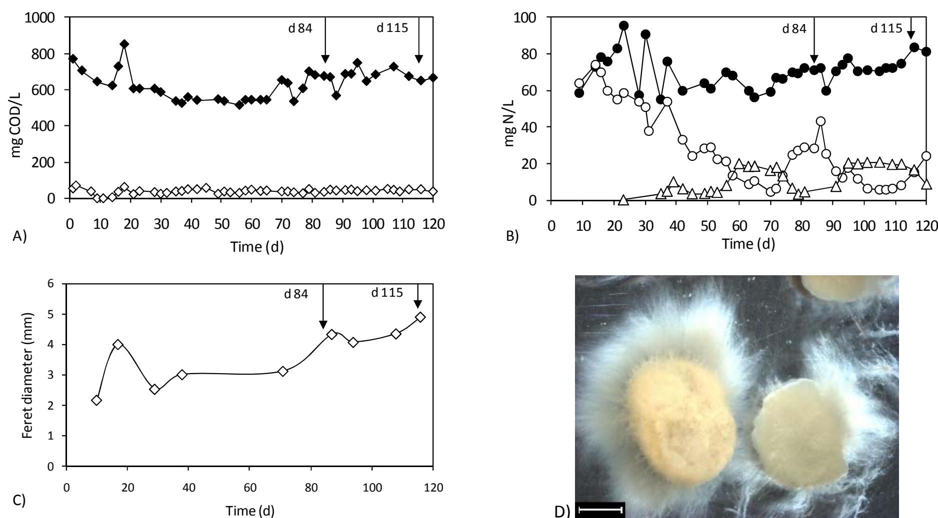


Figure 8.7. Performance of R3 and sampling dates (days 84 and 115) indicated with arrows. A) Organic matter concentration (mg COD/L): COD_{inf} (◆), COD_{eff} (◇); B) nitrogen compounds concentrations (mg N/L): NH_4^+-N_{inf} (●),NH_4^+-N_{eff} (○), NO_2^--N_{eff} (△) in the reactor. C) Evolution of the feret diameter (mm) and breakage episodes indicated with dotted arrows. D) Picture of the granules on day 116 with the bar indicating 2 mm.

The operation of this reactor was also marked by a problem with the air diffusers on day 65 of operation that provoked an insufficient shear stress in the reactor and low dissolved oxygen concentration in the bulk liquid. This event caused the outgrowth of filamentous

structures on the surface of the granules. On day 71 the mean feret diameter was of 3.1 mm with a slight increase up to 4.9 mm on day 115 (Figure 8.7C).

Observations made by the use of FISH

When the visualization of the biomass samples collected on days 84 and 115 from reactor R3 was performed filamentous bacteria were observed in both cases.

After the application of the probes for the identification of *Proteobacteria* classes, positive results were obtained only with probe Bet42a for *Betaproteobacteria,* but not for all of the filamentous bacteria observed with probe $EUB338_{mix}$. According to the existent data that related the phylogenetic affiliation of filamentous *Betaproteobacteria*, the possible organisms in the sample could be: *Curvibacter* (formerly *Aquaspirillum*), *Leptothrix discophora* or *Sphaerotilus natans* (Table 8.1).

After testing the specific probes available for each one of the mentioned organisms, Curvi997, LDI and SNA (Table 8.1), positive results were only obtained after the application of probe SNA for *Sphaerotilus natans* (Figure 8.8A). Furthermore its abundance in the sample was high and the characteristics of the observed filaments in the sample corresponded with those reported for the *S. natans* morphotype (straight or bent sheathed filaments) that in the present case rarely exceeded the 25 μm long (Figure 8.8B). The comparison between samples from day 84 and 115 indicates that the abundance of filamentous bacteria in both operating days was similar.

For many years, *Sphaerotilus* was believed to be the main causative organism of bulking in activated sludge systems (Eikelboom, 1975). Nowadays it is known that it has been frequently misidentified together with *Leptothrix* sp. It appears occasionally in plants with nutrient deficiency (van der Waarde *et al.*, 2002; Nielsen *et al.*, 2009b). *Sphaerotilus* are obligate aerobic organisms, able to consume a wide variety of carbon sources, organic acids, sugars, alcohols and amino acids (Nielsen *et al.*, 2009b). In contrast to most *Leptothrix* strains, *Sphaerotilus* is able to assimilate relatively high concentrations of substrates from which it synthesizes considerable amounts of poly-β-hydroxybutyrate (PHB) and polysaccharides. The synthesis of both reserve compounds is stimulated by a high carbon/nitrogen ratio in the medium or by oxygen deficiency (Spring, 2006).

The remainder fraction of filamentous bacteria was identified after the application of probe CFX1223. The filaments of *Chloroflexi* bacteria had a length between 15 and 25 μm and their abundance in this reactor was higher than in the case of R1 as it can be seen in Figure 8.8C and Figure 8.2D.

S. natans bacteria were found to be present in the inoculum used for the start up of the reactor, although the percentage in the sample was much lower than that observed on day

115 of operation. The comparison between samples from day 84 and 115 indicates that the abundance of filamentous bacteria in both operating days was similar.

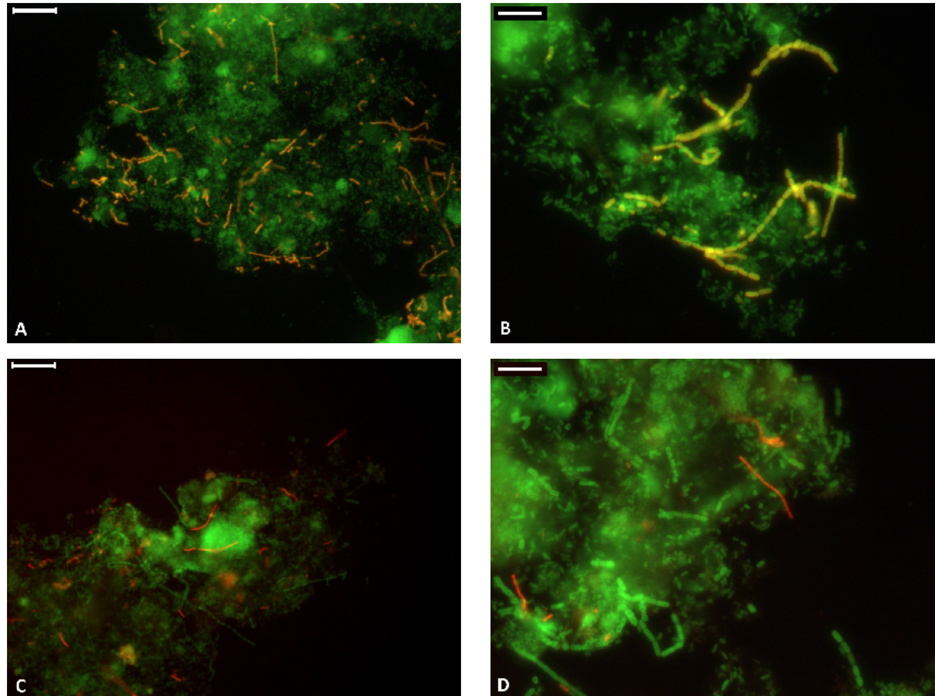


Figure 8.8. FISH images of filamentous bacteria from R3 on day 115. A) and B) SNA (Cy3) and $EUB338_{mix}$ (FITC). The bar indicates 25 µm and 10 µm, respectively. C) CFX1223 (Cy3) and $EUB338_{mix}$ (FITC). The bar indicates 25 µm and 10 µm, respectively.

One of the singularities of this system relies in the existence of another reactor operated under the same feeding conditions but without the addition of the coagulant and the floculant in the feeding (R3b). Even though the development of the granules was similar in both cases, the system R3b was characterized by slightly smaller size of the granules, and without the excessive outgrowth of filaments on their surface (Figure 8.9).

This difference was confirmed also under a microscopic point of view. In spite of the use of the same inoculum, neither *S. natans* nor *Chloroflexi* were observed when a sample of day 114 collected from reactor R3b was analyzed.

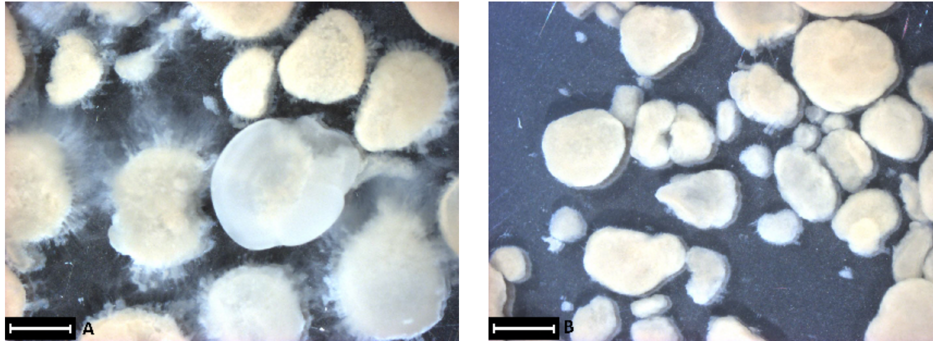


Figure 8.9. Images of granules on day 108: A) from the reactors R3 and B) R3b. The bar represents 2 mm.

Therefore it is clear that microbial populations developed in this system depended not only on the inoculum and the operational conditions but also on the addition of the coagulant and the flocculant. The coagulants are normally positively charged and they are designed to neutralize the repulsive electrical charges (typically negative) surrounding particles. Their presence favours the creation of flocs, while the flocculants facilitate the agglomeration or aggregation of the coagulated particles to form larger floccules. The addition of both reagents could retain filamentous bacteria in the system, and the posterior operational conditions led to their development.

8.4.4. Conditions for filamentous appearance

Several hypotheses to explain bulking and foaming in activated sludge were formulated by researchers in the hope of finding a general solution for this problem (Martins *et al.*, 2004). Unfortunately, none of them led to a definitive explanation but they can be used to explore the link with aerobic granules.

From the observations made by the FISH technique in the periods before and after the breakage of the granules in the studied reactors, it seems that their destabilization could be associated to a certain extent to the excessive growth of filamentous bacteria. Therefore, an interesting issue would be to research what factors promoted the development of the different filamentous populations in the aerobic granules.

The diffusion based theory connected the excessive growth of filamentous microorganisms with substrate diffusional resistance inside biological flocs (Kappeler and Gujer, 1994). It would also imply that under non-bulking process conditions filamentous bacteria can still be present inside the floc and if substrate limitation occurs they will then quickly grow out of the floc. In this case the filamentous morphology gives the organisms an ecological advantage. This theory would explain the presence of filamentous organism in the surface of the aerobic granules. From the pictures of the granules taken with the stereomicroscope (Figure 8.1D, Figure 8.4D, Figure 8.7D) the usual abundance of filamentous

structures on the surface of the granules is clearly observed. This fact has also been recently related to the presence of suspended solids or colloidal substrates in the feeding (de Kreuk *et al.*, 2010) and a way for filamentous bacteria to reach substrates. In addition filamentous growth has been shown as an also effective strategy of bacteria to exceed the size limit of particles edible by protozoa, thereby allowing them to escape from grazing (Sommaruga and Psenner, 1995).

The kinetic selection theory states that in systems where the substrate concentration is high, like in plug-flow reactors or SBR systems, the filamentous bacteria should be suppressed since their growth rate is expected to be lower than that for floc forming bacteria (Chudoba *et al.*, 1973). In this way the alternation of feast and famine periods is used to promote the formation of aerobic granules selecting for floc forming microorganisms (McSwain *et al.*, 2004). Another reason for the use of feast and famine periods is based on the ability of the floc forming bacteria to rapidly store the organic matter as glycogen, lipids or PHA under high organic substrate concentrations. This ability presumably provides an extra advantage to non-filamentous bacteria in dynamic systems, and it is thought that the formed biomass is absent of filamentous bacteria. However, it has been recently observed that certain filamentous organisms can store substrates to be metabolized for energy generation or protein production during the famine periods, which would represent a strong selective advantage in competition with other filamentous and non-filamentous bacteria. This ability could be especially advantageous under the feast-famine regimes (Martins *et al.*, 2004) encountered for example in the aerobic granular SBRs. This could be one of the reasons for the predominance of *Sphaerotilus natans* in R3 and *Meganema perideroedes* in R2, both having the storage ability.

Other possible causes of outgrowth of filamentous bacteria in aerobic granular sludge SBRs are dissolved oxygen and nutrient deficiency, long SRT values and temperature shifts (Liu and Liu, 2006). Therefore, because of the cyclic operation of aerobic granular sludge SBR and peculiarities of aerobic granules, several of these conditions can be present simultaneously and they can result in progressive development of filamentous growth in aerobic granular sludge SBR.

Two different sources can be explored in order to determine the origin of the filamentous bacteria in R1, R2 and R3, one is the inoculum and the other is the incoming wastewater. In the case of *Thiothrix* and *Meganema* bacteria, from reactors R1 and R2, they were not observed in the reference samples, whereas *S. natans,* from R3, was detected in the inoculum. Therefore, it is clear that the operational conditions of each system promoted the establishment and further development of filamentous bacteria that were present in each one of the feedings. The different sources of origin of the treated wastewater could be also responsible for the different species detected in the reactors.

However, from the results presented in Table 8.3 and from the evolution of the operational conditions of the reactors and the physical properties of the biomass it is difficult to assess which was the determinant factor for the excessive growth of each one of the filamentous species and the breakage of the granules in R1 and R2.

High solid concentrations, over 6 g VSS/L, were measured in R1 and R2, however, the concentrations achieved in R3 were around 2.5 g VSS/L. As a result of the biomass developed in the system and the fed OLR, the values of the F/M ratio were different in each reactor. Reactor R3 was operated under a high F/M ratio, and this was reported as one of the possible factors that causes the growth of *S. natans*. However, R1 and R2 were operated under such F/M values that are favourable for the growth of most of the filamentous species (Jenkins *et al.*, 2003). The increase of the F/M ratio between the two selected operating days could be favour the development of *Thiothrix* and *Meganema* species.

The increase of the OLR treated by the aerobic granules also caused the increase of their feret diameter. In R1 and R3 the feret diameter increased up to values of 5.8 and 4.9 mm in both selected operational days, while in the case of R2, although a feret diameter of 2.4 mm was measured on day 192, it reached a value of 11.0 mm on day 226, when the granules broke up. Therefore, a high OLR and feret diameter could hinder the dissolved oxygen transfer from the bulk liquid to the inner zones of the granules and promote the growth of filamentous bacteria and the breakage of the granules.

Table 8.3. Physical properties and operational conditions of the aerobic granular reactors near each sampling day.

	R1		**R2**		**R3**	
	day 268	**day 344**	**day 192**	**day 263**	**day 84**	**day 115**
$VSS_{reactor}$ (g/L)	6.7	7.3	10.8	6.5	2.0	2.7
SVI (mL/g TSS)	56.5	79.5	39.5	55.4	116.6	74.1
Feret diameter (mm)	4.9	5.8	2.4	n.d.	4.1	4.9
$OLR_{treated}$ (kg COD_s/m^3 d)	2.10	3.11	2.10	2.94	2.55	2.51
$NLR_{treated}$ (kg N/m^3 d)	0.12	0.09	0.02	0.07	0.17	0.19
F/M (kg COD_s/(kg VSS d))	0.31	0.42	0.19	0.51	1.27	0.95
SRT (d)	51	10	14	5	5	5
Organism observed and abundance	*Thiotrix* (+) *Chloroflexi* (+)	*Thiotrix* (+++) *Chloroflexi* (+)	*Meganema* (++) *Fungi* (++)	*Meganema* (+++) *Fungi* (++)	*S. natans* (+++) *Chloroflexi* (+)	*S. natans* (+++) *Chloroflexi* (+)

n.d.: not determined due to the breakage

Abundance estimation: average number of filaments in 10 pictures taken at 400x: +, (less than 10), ++ (10 - 35), +++ (more than 35).

Observed changes in SVI of biomass from R1 and R2 could be also related with the feret diameter and the growth of filamentous bacteria in the surface of the granules that would increase the fraction of voids when the biomass settled. Observed changes in R3 are not relevant, since the SVI fluctuated between 71 to 123 g VSS/L during the whole operational period.

From this study, the physiological traits of the filamentous bacteria in granules could not be deduced unless the specific FISH probes of each bacterium were used in concert with *in situ* microautoradiography to determine specific aspects of their phenotype (Lee *et al.*, 1999).

8.5. CONCLUSIONS

The use of the FISH technique provided an accurate identification and abundance estimation of filamentous microorganism from three different aerobic granular systems operated under different conditions: OLR, NLR, feeding composition, etc. Detected species were different in each case and therefore, these differences can be attributed to the kind of treated wastewater and the operational conditions. It could be checked in R1 and R2 that filamentous bacteria were not present during first months of operation, so their origin could be related with the treated wastewater. The origin of filamentous bacteria from R3 was the inoculum.

Thiothrix and *Chloroflexi* bacteria were identified in the reactor fed with the effluent produced in a fish canning industry (R1). *Meganema* bacteria and fungi were identified in the reactor fed with wastewater from a plant processing marine products with a previous physical-chemical treatment (R2). *Sphaerotilus natans* and *Chloroflexi* bacteria were present in the reactor that was fed with a synthetic medium with sodium acetate as carbon source supplemented with a residual quantity of coagulant and flocculant (R3).

Although filamentous bacteria seem to be ubiquitous in aerobic granules and to form part of the granular structure, when the operational conditions were favourable they could develop in such an extent that provoked the destabilisation of the granules. There are hypothesis to explain the factor that favor the development of filamentous bacteria in activated sludge processes that can be applied to aerobic granules, but from the comparison of the performance of the three reactors which one was the determinant factor was not clear.

8.6. REFERENCES

Amann R., Binder B.J., Olson R.J., Chisholm S.W., Devereux R. and Stahl D.A. (1990). Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial-populations. *Applied and Environmental Microbiology,* 56(6), 1919-1925.

APHA-AWWA-WPCF (2005) Standard methods for the examination of water and wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Baschien C. (2003) Development and evaluation of rRNA targeted in situ probes and phylogenetic relationships of freshwater fungi, Technical University of Berlin, Berlin.

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.

Bjornsson L., Hugenholtz P., Tyson G.W. and Blackall L.L. (2002). Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal 9. *Microbiology,* 148(8), 2309-2318.

Campos J.L., Figueroa M., Mosquera-Corral A. and Mendez R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering,* 1, 136-151.

Chudoba J., Grau P. and Ottova V. (1973). Control of activated sludge filamentous bulking II. Selection of microorganisms by means of a selector. *Water Research,* 7(10), 1389-&.

Daims H., Bruhl 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(3), 434-444.

de Kreuk M.K., Kishida N., Tsuneda S. and van Loosdrecht M.C.M. (2010). Behavior of polymeric substrates in an aerobic granular sludge system. *Water Research,* 44(20), 5929-5938.

Eikelboom D. (2000) Process control of activated sludge plants by microscopic investigation, IWA Publishing, London.

Eikelboom D.H. (1975). Filamentous organisms observed in activated sludge. *Water Research,* 9(4), 365-388.

Figueroa M., Mosquera-Corral A., Campos J.L. and Mendez R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. *Water Science and Technology,* 58(2), 479-485.

Gich F., Garcia-Gil J. and Overmann J. (2001). Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. *Archives of Microbiology,* 177(1), 1-10.

Jenkins D., Richard M.G. and Daigger G.T. (2003) Manual on the causes and control of activated sludge bulking, foaming, and other solids separation problems, IWA Publishing, London.

Kanagawa T., Kamagata Y., Aruga S., Kohno T., Horn M. and Wagner M. (2000). Phylogenetic analysis of and oligonucleotide probe development for Eikelboom Type 021N filamentous bacteria isolated from bulking activated sludge. *Applied and Environmental Microbiology,* 66(11), 5043-5052.

Kappeler J. and Gujer W. (1994). Development of a mathematical model for "aerobic bulking". *Water Research,* 28(2), 303-310.

Kindaichi T., Ito T. and Okabe S. (2004). Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. *Applied and Environmental Microbiology,* 70(3), 1641-1650.

Kragelund C., Nielsen J.L., Thomsen T.R. and Nielsen P.H. (2005). Ecophysiology of the filamentous Alphaproteobacterium Meganema perideroedes in activated sludge. *FEMS Microbiology Ecology,* 54(1), 111-112.

Kragelund C., Levantesi C., Borger A., Thelen K., Eikelboom D., Tandoi V., Kong Y., Waarde J.v.d., Krooneman J., Rossetti S., Thomsen T.R. and Nielsen P.H. (2007). Identity, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. *FEMS Microbiology Ecology,* 59(3), 671-682.

Lee N., Nielsen P.H., Andreasen K.H., Juretschko S., Nielsen J.L., Schleifer K.H. and Wagner M. (1999). Combination of fluorescent in situ hybridization and microautoradiography-a new tool for structure-function analyses in microbial ecology. *Applied and Environmental Microbiology,* 65(3), 1289-1297.

Levantesi C., Beimfohr C., Geurkink B., Rossetti S., Thelen K., Krooneman J., Snaidr J., van der Waarde J. and Tandoi V. (2004). Filamentous Alphaproteobacteria associated with bulking in industrial wastewater treatment plants. *Systematic and Applied Microbiology,* 27(6), 716-727.

Liu J.R., Burrell P., Seviour E.M., Soddell J.A., Blackall L.L. and Seviour R.J. (2000). The filamentous bacterial morphotype 'Nostocoida limicola' I contains at least two previously described genera in the low G+C gram positive bacteria. *Systematic and Applied Microbiology,* 23(4), 528-534.

Liu J.R., McKenzie C.A., Seviour E.M., Webb R.I., Blackall L.L., Saint C.P. and Seviour R.J. (2001). Phylogeny of the filamentous bacterium 'Nostocoida limicola' III from activated sludge. *International Journal of Systematic and Evolutionary Microbiology,* 51(Pt 1), 195-202.

Liu J.R. and Seviour R.J. (2001). Design and application of oligonucleotide probes for fluorescent in situ identification of the filamentous bacterial morphotype Nostocoida limicola in activated sludge. *Environmental Microbiology,* 3(9), 551-560.

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.

Loy A., Maixner F., Wagner M. and Horn M. (2007). probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Research,* 35(suppl_1), D800-D804.

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(4), 593-600.

Martins A.M.P., Pagilla K., Heijnen J.J. and van Loosdrecht M.C.M. (2004). Filamentous bulking sludge--a critical review. *Water Research,* 38(4), 793-817.

Matsumoto S., Katoku M., Saeki G., Terada A., Aoi Y., Tsuneda S., Picioreanu C. and Van Loosdrecht M.C.M. (2010). Microbial community structure in autotrophic nitrifying granules characterized by experimental and simulation analyses. *Environmental Microbiology,* 12(1), 192-206.

McSwain B.S., Irvine R.L. and Wilderer P.A. (2004). Effect of intermittent feeding on aerobic granule structure. *Water Science and Technology,* 49(11-12), 19-25.

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.

Nicolau A., Dias N., Mota M. and Lima N. (2001). Trends in the use of protozoa in the assessment of wastewater treatment. *Research in Microbiology,* 152(7), 621-630.

Nielsen H., Daims H. and Lemmer H. (2009a) FISH handbook for biological wastewater treatment, IWA Publishing, London, UK.

Nielsen P.H., De Muro M.A. and Nielsen J.L. (2000). Studies on the in situ physiology of Thiothrix spp. present in activated sludge. *Environmental Microbiology,* 2(4), 389-398.

Nielsen P.H., Kragelund C., Seviour R.J. and Nielsen J.L. (2009b). Identity and ecophysiology of filamentous bacteria in activated sludge. *FEMS Microbiology Reviews,* 33(6), 969-998.

Nittami T., McIlroy S., Seviour E.M., Schroeder S. and Seviour R.J. (2009). Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III Defluviicoccus. *Systematic and Applied Microbiology,* 32(7), 480-489.

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(5), 579-584.

Schade M., Beimfohr C. and Lemmer H. (2002). Phylogenetic and physiological characterization of a "Nostocoida limicola"-like organism isolated from activated sludge. *Water Science and Technology,* 46(1-2), 91-97.

Seviour E.M., McIlroy S. and Seviour R. (2010). Descriptions of activated sludge organisms in *Microbial ecology of activated sludge*. Seviour, R. and Nielsen, P.H. (eds), IWA publishing, London.

Snaidr J., Beimfohr C., Levantesi C., Rossetti S., van der Waarde J., Geurkink B., Eikelboom D., Lemaitre M. and Tandoi V. (2002). Phylogenetic analysis and in situ identification of "Nostocoida limicola"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. *Water Science and Technology,* 46(1-2), 99-104.

Sommaruga R. and Psenner R. (1995). Permanent presence of grazing-resistant bacteria in a hypertrohic lake. *Applied and Environmental Microbiology,* 61, 3457-3459.

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

Spring S. (2006). The genera *Lepthothrix* and *Sphaerotilus* in *The Prokaryotes: Ecophysiology and Biochemistry, vol. 5*. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. (eds), Springer, New York.

Tay J.H., Liu Q.S. and Liu Y. (2001). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *Journal of Applied Microbiology,* 91(1), 168-175.

Thomsen T.R., Nielsen J.L., Ramsing N.B. and Nielsen P.H. (2004). Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. *Environmental Microbiology,* 6(5), 470-479.

Thomsen T.R., Blackall L.L., de Muro M.A., Nielsen J.L. and Nielsen P.H. (2006). Meganema perideroedes gen. nov., sp. nov., a filamentous alphaproteobacterium from activated sludge. *International Journal of Systematic and Evolutionary Microbiology,* 56(8), 1865-1868.

Tijhuis L., van Loosdrecht M.C.M. and Heijnen J.J. (1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnology and Bioengineering,* 44(5), 595-608.

Val del Río A., Figueroa M., Arrojo B., Mosquera-Corral A., Campos J.L., García-Torriello G. and Mendez R. (accepted). Aerobic granular SBR systems applied to the treatment of industrial effluents. *Journal of Environmental Management*.

van der Waarde J., Krooneman J., Geurkink B., van der Werf A., Eikelboom D., Beimfohr C., Snaidr J., Levantesi C. and Tandoi V. (2002). Molecular monitoring of bulking sludge in industrial wastewater treatment plants. *Water Science and Technology,* 46(1-2), 551-558.

Wagner M., Amann R., Kampfer P., Assmus B., Hartmann A., Hutzler P., Springer N. and Schleifer K.H. (1994). Identification and in-situ detection of Gram-negative filamentous bacteria in activated-sludge. *Systematic and Applied Microbiology,* 17(3), 405-417.

Weber S.D., Ludwig W., Schleifer K.H. and Fried J. (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Applied and Environmental Microbiology,* 73(19), 6233-6240.

Weber S.D., Hofmann A., Pilhofer M., Wanner G., Agerer R., Ludwig W., Schleifer K.H. and Fried J. (2009). The diversity of fungi in aerobic sewage granules assessed by 18S rRNA gene and ITS sequence analyses. *FEMS Microbiology Ecology,* 68(2), 246-254.

Williams T.M. and Unz R.F. (1985). Filamentous sulfur bacteria of activated sludge: characterization of Thiothrix, Beggiatoa, and Eikelboom type 021N strains. *Applied and Environmental Microbiology,* 49(4), 887-898.

Yamada T., Sekiguchi Y., Imachi H., Kamagata Y., Ohashi A. and Harada H. (2005). Diversity, localization, and physiological properties of filamentous microbes belonging to Chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules. *Applied and Environmental Microbiology,* 71(11), 7493-7503.

Conclusiones generales

En el presente trabajo de investigación, se evaluó la puesta en marcha y operación de reactores secuenciales (SBR) aerobios con biomasa granular para el tratamiento de efluentes de la industria conservera y de producción de ganado porcino. Muestras de la biomasa granular obtenida en estas unidades se analizaron con el fin de identificar las principales poblaciones bacterianas responsables de los procesos de eliminación de materia orgánica y de nitrógeno de los citados efluentes.

Las conclusiones más importantes obtenidas en este trabajo se agrupan en los apartados que se describen brevemente a continuación:

1. ***Tratamiento de efluentes procedentes de la industria agroalimentaria en sistemas granulares aerobios:***

Los sistemas granulares aerobios mostraron su viabilidad para el tratamiento de los efluentes seleccionados. Se consiguieron tratar altas velocidades de carga orgánica (VCO) y nitrogenada (VCN).

En el reactor granular que trató el efluente de una industria de conservera se alcanzaron valores de VCO de 2,8 kg DQO/(m^3 d) y de VCN de 0,15 kg N/(m^3 d). En esta unidad, la presencia de concentraciones de sal de 10 g NaCl/L pudo causar el retraso en la formación de los gránulos. Las variaciones en la salinidad durante la operación del reactor no afectaron a los procesos biológicos una vez que los gránulos estaban formados, aunque se observaron cambios en la morfología de estos últimos en la etapa en que la concentración de sal se redujo a 0.06 g NaCl/L.

En el sistema tratando la fracción líquida de los purines de cerdo fue posible alcanzar la eliminación de materia orgánica y nitrógeno mediante el proceso de nitrificación y desnitrificación simultanea (SND) cuando las cargas aplicadas al sistema fueron de 4,4 kg DQO/(m^3 d) y de 0,83 kg N/(m^3 d). Esto supuso unas eficacias de eliminación simultánea del 87% de materia orgánica y 70% de nitrógeno. La eficacia de este proceso simultáneo se vio reducida cuando se aplicó una VCO de 7,0 kg DQO/(m^3 d) y una VCN de 1,26 kg N/(m^3 d).

Se concluye por tanto que la eliminación conjunta de materia orgánica y nitrógeno está afectada por los valores de carga orgánica aplicada, de modo que si se supera un valor máximo de carga orgánica la eliminación de nitrógeno disminuye o desaparece.

2. *Efecto de la relación de intercambio volumétrico (VER):*

La reducción de la relación de intercambio volumétrico del 50% al 6%, con el fin de tratar la fracción líquida del purín de cerdo sin diluir, ni afectó a la operación del sistema ni a las propiedades físicas de los gránulos formados. Esta reducción permitió tratar elevadas concentraciones de materia orgánica y nitrógeno. La concentración de sólidos en el influente aumentó hasta alcanzar valores de 4,9 g SSV/L, lo que causó el desarrollo de biomasa en forma dispersa. Ésta no se eliminó totalmente del sistema debido al pequeño volumen de líquido retirado en cada ciclo, arrastrándose además gránulos embebidos en ella, lo que generó un descenso de la concentración de biomasa en el reactor de 16,0 a 4,7 g SSV/L.

Se concluye que la reducción del intercambio volumétrico provoca la modificación no sólo de la concentración de biomasa retenida en el sistema sino también de sus propiedades físicas.

3. *Característica físicas de la biomasa granular aerobia:*

En los reactores aerobios granulares se obtuvieron elevadas concentraciones de biomasa, con valores entre 10 y 15 g SSV/L. Los gránulos aerobios desarrollados se caracterizaron por sus buenas propiedades de sedimentabilidad, con valores del índice volumétrico de lodos alrededor de 30 mL/g SST y densidades de hasta 60 g $SSV/L_{gránulo}$ en el caso del reactor tratando un efluente generado en una industria conservera.

En los dos sistemas granulares se observaron episodios de ruptura de los gránulos y pérdidas de biomasa cuando ésta alcanzaba el nivel de vaciado del reactor, que aunque produjeron variaciones en la concentración de biomasa en los sistemas, no causó su desestabilización.

En el reactor granular tratando la fracción líquida de purines de cerdo se observó la coexistencia entre biomasa dispersa y biomasa granular, pero el equilibrio alcanzado es delicado puesto que los gránulos se quedaban retenidos en la matriz de biomasa dispersa.

Así, la biomasa granular se consigue fácilmente en condiciones aerobias en sistemas tratando efluentes agroalimentarios. Las cargas aplicadas así como las condiciones de operación le confieren distintas propiedades no siempre predicibles.

4. *Post-tratamiento de la fracción líquida de purines en un sistema CANON:*

El nitrógeno de la fracción liquida de los purines de cerdo, pre-tratados aeróbicamente en un reactor granular aerobio, se eliminó de forma satisfactoria en un reactor CANON operado a 20 °C. El uso de pulsos favoreció el control del oxígeno disuelto en el medio líquido para mantener las condiciones microaerobias. Se aplicó una carga de 0,60 kg N/(m^3 d) y se obtuvo una eficacia de eliminación del 75%, cuando la concentración de nitrógeno era de 300 mg $N\text{-}NH_4^+$/L.

La presencia de la fracción de materia orgánica lentamente o no biodegradable no afectó a la operación del sistema CANON debido al desarrollo de la biomasa en dos fases diferentes. Una de ellas estaba formada por gránulos de 2,6 mm de diámetro, principalmente de bacterias anammox, y la otra por biomasa en forma dispersa, principalmente bacterias oxidantes de amonio y hetetrótrofas. El desarrollo de estas dos fases minimizó la competición de las bacterias oxidantes de amonio, anammox y heterótrofas por los sustratos comunes.

Los resultados globales indicaron que un sistema combinado compuesto por un reactor granular aerobio y un sistema CANON permitió eliminar cargas de 2,12 kg DQO/(m^3 d) y 0,30 kg N/(m^3 d). Sin embargo en una única undad CANON se trataron cargas de 0,46 kg N/(m^3 d) lo que indica que éste no era el proceso limitante.

5. Caracterización de poblaciones microbianas:

Las técnicas de biología molecular como FISH, DGGE o la clonación resultaron ser herramientas útiles para la identificación de las principales poblaciones microbianas implicadas en la eliminación de la materia orgánica y nitrógeno en distintos tipos de reactores. También permitieron visualizar las bacterias filamentosas presentes en los gránulos formados.

5.1. Implicadas en la eliminación de la materia orgánica

Las principales poblaciones bacterianas implicadas en la eliminación de materia orgánica están presentes en los gránulos obtenidos en condiciones aerobias y experimentan cambios con el tiempo de operación de los sistemas. Las poblaciones bacterianas identificadas están comúnmente presentes en muestras de lodos activos, lo que significa que no se ha detectado una población especial dedicada a formar gránulos.

En el reactor que trató el efluente de la industria conservera se identificaron mediante clonación y DGGE, distintos miembros de las subclases *Alfa-*, *Beta-* y *Gammaproteobacteria* y los filos *Bacteroidetes* y *Chloroflexi*. La agrupación por semejanza de los perfiles de DGGE obtenidos de muestras de biomasa tomadas en distintos días de operación indicó que en las primeras etapas de operación del sistema existían bacterias relacionadas con el filo *Bacteroidetes* y *Thauera*, mientras que al final de la operación se desarrollaron *Thiothrix* y *Chloroflexi*.

El uso de sondas específicas de FISH permitió visualizar *in situ* la mayoría de las poblaciones de bacterias heterótrofas previamente identificadas. Se observaron las bacterias pertenecientes al filo *Bacteroidetes*, la familia *Comamonadaceae* y los géneros *Zoogloea* y *Thauera*. Además, se comprobó la distinta morfología de las bacterias *Bacteroidetes* en dos muestras diferentes del reactor.

Debido a las características de la alimentación del sistema CANON, con presencia de materia orgánica, también se desarrollaron bacterias heterótrofas, que fueron identificadas por clonación como pertenecientes al filo *Bacteroidetes*, y las familias *Comamonadacea*e y *Rhodocyclaceae.*

5.2. Implicadas en la eliminación de nitrógeno

En estos sistemas alimentados con altas concentraciones de amonio, las bacterias *Nitrosomonas* fueron las bacterias oxidantes de amonio más abundantes.

El uso de sondas específicas de FISH permitió visualizar *in situ* las poblaciones de bacterias oxidantes de amonio *Nitrosomonas* como responsables de la oxidación de nitrógeno en los sistemas estudiados. En el reactor granular tratando el efluente de la industria conservera, por ser un sistema meramente heterótrofo, la cantidad de bacterias oxidantes de amonio rondó el 8% mientras que en el sistema autótrofo CANON fue de 35%.

Mediante la aplicación de clonación se identificaron las bacterias anammox responsables de la oxidación anaerobia del amonio en el proceso CANON. Estas bacterias pertenecían a las especies *C*. "Brocadia fulgida" y *C*. "Brocadia anammoxidans". Mediante la técnica FISH se estimó su abundancia en un 30%. Aunque la coexistencia de distintas especies anammox en un mismo sistema no es frecuente, en este caso pudo estar asociada a las condiciones de operación del reactor.

5.3. Presencia de bacterias filamentosas

En tres reactores granulares aerobios que trataban efluentes de la industria conservera y de procesado de productos marinos y un efluente sintético, respectivamente, se observaron organismos de tipo filamentoso. Debido a las características propias de cada uno de los sistemas se favoreció el desarrollo de especies diferentes en periodos de operación diferentes. El estudio de periodos previos indicó que su origen es la propia agua residual tratada o el inóculo utilizado para el arranque del reactor.

En el reactor tratando el agua residual de la industria conservera se visualizaron bacterias *Thiothrix* y *Chloroflexi*. En el reactor tratando agua de una industria de procesado de productos marinos se identificaron no solo bacterias del tipo *Meganema perideroedes* sino también organismos eucariotas del reino Hongos. En el reactor alimentado con un medio sintético suplementado con compuestos coagulantes y floculantes se observaron bacterias del tipo *Chloroflexi* y *Sphaerotilus natans.*

A partir del análisis de las condiciones de operación de cada uno de los sistemas se postuló que el desarrollo excesivo de organismos filamentosos serían los causantes de la ruptura de los gránulos aerobios.

ALGUNAS REFLEXIONES Y PERSPECTIVAS FUTURAS

Es preciso resaltar que no existe una opción única para el tratamiento de las aguas residuales y que los factores que determinan que un sistema sea mejor que otro son múltiples. Los sistemas granulares aerobios serán adecuados en aquellas situaciones en las que el objetivo sea la eliminación de la materia orgánica y la reducción del contenido de nitrógeno y que debido a las limitaciones de espacio se requiera la implantación de sistemas más compactos. El sistema CANON operado a bajas temperaturas surge como post-tratamiento de corrientes con una pequeña relación carbono/nitrógeno y en las que debido a la legislación vigente se hace necesario reducir el contenido de nitrógeno.

A nivel laboratorio es necesario seguir trabajando en la optimización de la distribución de los ciclos de operación de los sistemas secuenciales, de forma que se consiga una mayor eficacia en la eliminación de nitrógeno. Esta optimización debe hacerse para cada tipo de agua residual a tratar, ya que su composición es determinante en la operación del reactor. Modificaciones en la concentración del oxígeno o la longitud del ciclo podrían influir en los perfiles de concentración de cada uno de los sustratos.

Los sistemas granulares aerobios y el proceso CANON han mostrado ser muy eficientes a nivel laboratorio en cuanto a la eliminación de materia orgánica y nitrógeno de distintas corrientes de origen industrial por lo que su implementación a escala industrial es prometedora.

Sin embargo aún quedan por optimizar distintos aspectos que deberían ser considerados para su implantación:

1. Establecer sistemas de recirculación de aire en los sistemas aerobios para poder minimizar los gastos de aireación, ya que se requieren caudales elevados para mantener la biomasa en suspensión y alcanzar el estrés hidrodinámico que promueve la formación de gránulos.
2. Necesidad de establecer purgas periódicas de biomasa para evitar eventos de lavado de la misma debidos al crecimiento de los gránulos, controlar el tiempo de retención de sólidos, y el acoplamiento de un pequeño sedimentador para reducir los sólidos en el punto de vertido.

Aunque el uso de técnicas específicas para la identificación de poblaciones como FISH, DGGE o clonación son en la actualidad de difícil aplicación en las estaciones depuradoras de aguas residuales, el conocimiento que proporcionan puede ser de gran utilidad para el diseño y operación de los sistemas biológicos. Estos pueden dejar así de ser considerados como auténticas cajas negras en las que sólo importan las corrientes de entrada y salida del sistema.

Conclusións xerais

No presente traballo de investigación, avaliouse a posta en marcha e operación de reactores secuenciais (SBR) aerobios con biomasa granular para o tratamento de efluentes da industria conserveira e da produción de gando porcino. Mostras da biomasa granular obtida nestas unidades foron analizadas co fin de identificar as principais poboacións bacterianas responsables dos procesos de eliminación de materia orgánica e de nitróxeno dos citados efluentes.

Así, as conclusións máis importantes obtidas neste traballo agrúpanse nos apartados que se describen brevemente a continuación:

1. Tratamento de efluentes procedentes da industria agroalimentaria en sistemas granulares aerobios:

Os sistemas granulares aerobios mostraron a súa viabilidade para o tratamento dos efluentes seleccionados. Conseguíronse tratar altas velocidades de carga orgánica (VCO) e nitroxenada (VCN). No reactor granular que tratou o efluente dunha industria conserveira alcanzáronse valores de VCO de 2,8 kg DQO/(m^3 d) e de VCN de 0,15 kg N/(m^3 d).

Nesta unidade, a presenza de concentracións de sal de 10 g NaCl/L puido causar o atraso na formación dos gránulos. As variacións na salinidade durante a operación do reactor non afectaron aos procesos biolóxicos unha vez que os gránulos estaban formados, aínda que se observaron cambios na morfoloxía destes últimos na etapa en que a concentración de sal se reduciu a 0.06 g NaCl/L.

No sistema tratando a fracción líquida dos xurros de porco foi posible alcanzar a eliminación de materia orgánica e nitróxeno mediante o proceso de nitrificación e desnitrificación simultanea (SND) cando as cargas aplicadas ao sistema foron de 4,4 kg DQO/(m^3 d) e de 0,83 kg N/(m^3 d). Isto supuxo unhas eficacias de eliminación do 87% e 70% de materia orgánica e nitróxeno simultaneamente. A eficacia deste proceso simultáneo viuse reducida cando se aplicou unha VCO de 7,0 kg DQO/(m^3 d) e unha VCN de 1,26 kg N/(m^3 d).

Conclúese por tanto que a eliminación conxunta de materia orgánica e nitróxeno está afectada polos valores de carga orgánica aplicada, de modo que si se supera un valor máximo de carga orgánica a eliminación de nitróxeno diminúe ou desaparece.

2. *Efecto da relación de intercambio volumétrico (VER)*

A redución da relación de intercambio volumétrico do 50% ao 6% co fin de tratar a fracción líquida do xurro de porco sen diluír nin afectou á operación do sistema nin ás propiedades físicas dos gránulos formados. Esta redución permitiu tratar elevadas concentracións de materia orgánica e nitróxeno. A concentración de sólidos no influente aumentou ata alcanzar valores de 4,9 g SSV/L, o que causou o desenvolvemento de biomasa en forma dispersa. Esta non se eliminou totalmente do sistema debido ao pequeno volume de líquido retirado en cada ciclo e ademais arrastraba gránulos embebidos nela, o que xerou un descenso da concentración de biomasa no reactor de 16,0 a 4,7 g SSV/L.

Por tanto a redución do intercambio volumétrico provoca a modificación non só da concentración de biomasa retida no sistema senón tamén das súas propiedades físicas.

3. *Característica físicas da biomasa granular aerobia:*

Nos reactores aerobios granulares obtivéronse elevadas concentracións de biomasa, con valores entre 10 e 15 g SSV/L. Os gránulos aerobios desenvolvidos caracterizáronse polas súas boas propiedades de sedimentabilidad, con valores do índice volumétrico de lodos ao redor de 30 mL/g SST e densidades de ata 60 g SSV/$L_{gránulo}$ no caso do reactor tratando o efluente xerado nunha industria conserveira.

Nos dous sistemas granulares observáronse episodios de ruptura dos gránulos e perdas de biomasa cando esta alcanzaba o nivel de baleirado do reactor, que aínda que produciron variacións na concentración de biomasa nos sistemas, non causou a súa desestabilización.

A coexistencia entre biomasa dispersa e biomasa granular foi posible no reactor granular tratando a fracción líquida de xurros de porco, pero o equilibrio era delicado posto que os gránulos quedaban retidos na matriz de biomasa dispersa.

A biomasa granular conséguese facilmente en condicións aerobias en sistemas tratando efluentes agroalimentarios. As cargas aplicadas así como as condicións de operación confírenlle distintas propiedades.

4. *Post-tratamento da fracción líquida de xurros nun sistema CANON*

O nitróxeno da fracción liquida dos xurros de porco, pretratados aeróbicamente nun reactor granular aerobio, eliminouse de forma satisfactoria nun reactor CANON operado a 20 °C. O uso de pulsos favoreceu o control do osíxeno disolto no medio líquido para manter as condicións microaerobias. Aplicouse unha carga de 0,60 kg N/(m^3 d) e obtívose unha eficacia de eliminación do 75% cando a de nitróxeno era de 300 mg N-NH_4^+/L.

A presenza da fracción de materia orgánica lentamente ou non biodegradable non afectou á operación do sistema CANON debido ao desenvolvemento da biomasa en dúas fases diferentes. Unha delas estaba formada por gránulos de 2,6 mm de diámetro, principalmente de bacterias anammox, e a outra por biomasa en forma dispersa, principalmente bacterias oxidantes de amonio e hetetrótrofas. O desenvolvemento destas dúas fases minimizou a competición das bacterias oxidantes de amonio, anammox e heterótrofas polos substratos comúns.

Os resultados globais indicaron que un sistema combinado composto por un reactor granular aerobio e un sistema CANON permitiron eliminar cargas de 2,12 kg DQO/(m^3 d) e 0,3 kg N/(m^3 d). Sen embargo, nunha única unidade CANON tratáronse cargas de 0,46 kg/(m^3 d) o que indicou que este non era o proceso limitante.

5. Caracterización de poboacións microbianas:

As técnicas de bioloxía molecular como FISH, DGGE ou a clonación resultaron ser ferramentas útiles para a identificación das principais poboacións microbianas implicadas na eliminación da materia orgánica e nitróxeno en distintos tipos de reactores. Tamén permitiron visualizar as bacterias filamentosas presentes nos gránulos formados.

5.1. Implicadas na eliminación da materia orgánica

As principais poboacións bacterianas implicadas na eliminación de materia orgánica e que están presentes nos gránulos obtidos en condicións aerobias experimentan cambios co tempo de operación dos sistemas. As poboacións bacterianas identifiadas están comunmente presentes em mostras de lamas activas, o que significa que non se detectou unha poboación especial dedicada a formar os gránulos.

No reactor que tratou o efluente da industria conserveira identificáronse mediante clonación e DGGE, distintos membros das subclases *Alfa-*, Beta- e *Gammaproteobacteria* e os filums *Bacteroidetes* e *Chloroflexi*. A agrupación por semellanza dos perfís de DGGE obtidos de mostras de biomasa tomadas en distintos días de operaión indicou que nas primeiras etapas de operación do reactor existían bacterias relacionadas co filum *Bacteroidetes* e *Thauera*, mentres que ao final da operación desenvolvéronse *Thiothrix* e *Chloroflexi*.

O uso de sondas específicas de FISH permitiu visualizar *in situ* a maioría das poboacións de bacterias heterótrofas previamente identificadas. Observáronse as bacterias pertencentes ao filum *Bacteroidetes*, a familia *Comamonadaceae* e os xéneros *Zoogloea* e *Thauera*. Ademais, comprobouse a distinta morfoloxía das bacterias *Bacteroidetes* en dúas mostras diferentes do reactor.

Debido ás características da alimentación do sistema CANON, con presenza de materia orgánica, tamén se desenvolveron bacterias heterótrofas, que foron identificadas por

clonación como pertencentes ao filum *Bacteroidaceae*, e as familias *Comamonadaceae* e *Rhodocyclaceae*.

5.2. Implicadas na eliminación de nitróxeno

Nestos sistemas alimentados con altas concentracións de amonio, as bacterias *Nitrosomonas* foron as bacterias oxidantes de amonio máis abundantes.

O uso de sondas específicas de FISH permitiu visualizar *in situ* as poboacións de bacterias oxidantes de amonio *Nitrosomonas* como responsables da oxidación de nitróxeno no reactor tratando o efluente da industria conserveira e no sistema CANON. Por ser un sistema meramente heterótrofo, a cantidade de bacterias oxidantes de amonio no sistema granular roldou o 8% mentres que no sistema autótrofo CANON foi de 35%.

Mediante a aplicación de clonación identificáronse as bacterias anammox responsables da oxidación anaerobia do amonio no proceso CANON. Estas bacterias pertencían ás especies *C*. "Brocadia fulgida" e *C*."Brocadia anammoxidans". Mediante a técnica FISH estimouse a súa abundancia nun 30%.

A coexistencia de distintas especies anammox nun mesmo sistema non é frecuente e pued vir asociado ás condicións de operación do mesmo.

5.3. Presenza de bacterias filamentosas

En tres reactores granulares aerobios que trataban efluentes da industria de enlatado e de procesado de produtos mariños e un efluente sintético, respectivamente, observáronse organismos de tipo filamentoso. Debido ás características propias de cada un dos sistemas favoreceuse o desenvolvemento de especies diferentes en períodos de operación diferentes. O estudo de períodos previos indicou que a súa orixe é a propia auga residual tratada ou o inóculo utilizado para o arranque do reactor.

No reactor tratando a auga residual da industria conserveira visualizáronse bacterias *Thiothrix* e *Chloroflexi*. No reactor tratando auga dunha industria de procesado de produtos mariños identificáronse bacterias *Meganema perideroedes* e tamén se observaron organismos eucariotas do reino Fungos. No reactor alimentado cun medio sintético suplementado con compostos coagulantes e floculantes observáronse bacterias do tipo Chloroflexi e Sphaerotilus natans.

A partir da análise das condicións de operación de cada un dos sistemas postulouse que o desenvolvemento excesivo de organismos filamentosos serían os causantes da ruptura dos gránulos aerobios.

ALGUNHAS REFLEXIÓNS E PERSPECTIVAS FUTURAS

É preciso resaltar que non existe unha opción única para o tratamento das augas residuais e que os factores que determinan que un sistema sexa mellor que outro son múltiples. Os sistemas granulares aerobios serán adecuados naquelas situacións nas que o obxectivo sexa a eliminación da materia orgánica e a redución do contido de nitróxeno e que debido ás limitacións de espazo se requira a implantación de sistemas máis compactos. O sistema CANON operado a baixas temperaturas xorde como un post-tratamento de correntes cunha pequena relación carbono/nitróxeno e nas que debido á lexislación vixente faise necesario reducir o contido de nitróxeno.

A nivel laboratorio é necesario seguir traballando na optimización da distribución dos ciclos de operación dos sistemas secuenciais, de forma que se consiga unha maior eficacia na eliminación de nitróxeno. Esta optimización debe facerse para cada tipo de auga residual a tratar, xa que a súa composición é determinante na operación do reactor. Modificacións na concentración do osíxeno ou a lonxitude do ciclo poderían influír nos perfís de concentración de cada un dos substratos.

Os sistemas granulares aerobios e o proceso CANON mostraron ser moi eficientes a nivel laboratorio en canto á eliminación de materia orgánica e nitróxeno de distintas correntes de orixe industrial polo que a súa implementación a escala industrial é prometedora.

Con todo aínda quedan por optimizar distintos aspectos que deberían ser considerados para a súa implantación:

1. Establecer sistemas de recirculación de aire nos sistemas aerobios para poder minimizar os gastos de aireación, xa que se requiren caudais elevados para manter a biomasa en suspensión e alcanzar o estrés hidrodinámico que promove a formación de gránulos.

2. Necesidade de establecer purgas de biomasa para evitar eventos de lavado da mesma debidos ao crecemento dos gránulos e controlar o tempo de retención celular, e axuste dun pequeno sedimentador para reducir os sólidos no punto de vertedura.

Aínda que o uso de técnicas específicas para a identificación de poboacións como FISH, DGGE ou clonación parecen de difícil aplicación nas estacións depuradoras de augas residuais, o coñecemento que proporcionan pode ser de gran utilidade para o deseño e operación dos sistemas biolóxicos. Estes poden deixar así de ser considerados como auténticas caixas negras nas que só importan as correntes de entrada e saída do sistema.

General conclusions

In the present research work, the start up and operation of sequencing batch reactors (SBR) with aerobic granular biomass for the treatment of effluents from the fish canning industry and from the swine livestock production were evaluated. Samples of granular biomass obtained in these units were analysed with the aim of identifying the main bacterial populations responsible for the processes of organic matter and of nitrogen removal from the effluents of industrial origin.

The general conclusions of this work are grouped in the sections that are briefly described below:

1. Treatment of effluents from the agri-food industry in aerobic granular systems:

Aerobic granular systems showed their feasibility for the treatment of the selected effluents. High organic (OLR) and nitrogen loading rates (NLR) were successfully treated.

In the granular reactor that treated the effluent from a fish canning industry, values of OLR up to 2.8 kg COD/(m^3 d) and of NLR of 0.15 kg N/(m^3 d) were achieved. In this unit, the presence of salt concentrations up to 10 g NaCl/L could cause the delay in the formation of the granules. Salinity variations during the reactor operation did not affect the biological processes once the granules were formed, although changes in their morphology were observed in the last stage when the salt concentration was reduced to 0.06 g NaCl/L.

In the system treating the liquid fraction of swine slurry to achieve the organic matter and nitrogen removal by means of the simultaneous nitrification and denitrification (SND) process was possible when loads applied to the system were of 4.4 kg COD/(m^3 d) and of 0.83 kg N/(m^3 d). This implied simultaneous removal efficiencies of 87% and 70% in terms of organic matter and nitrogen, respectively. The efficiency of this simultaneous process was reduced when an OLR of 7.0 kg COD/(m^3 d) and a NLR of 1.26 kg N/(m^3 d) were applied.

So, the simultaneous organic matter and nitrogen removal is affected by the values of applied organic load, and if a maximum value of organic load is exceeded, the elimination of nitrogen diminishes or disappears.

2. Effect of the volumetric exchange ratio (VER)

The reduction of the volumetric exchange ratio from 50% to 6%, with the aim of treating the liquid fraction of the swine slurry without diluting, affected neither the operation of the system nor the physical properties of the formed granules. This reduction allowed treating high organic matter and nitrogen concentrations. The solids concentration in the influent increased up to values of 4.9 g VSS/L, what caused the development of biomass in dispersed form. This biomass was not totally removed from the system because of the small volume of withdrawn liquid in each cycle and besides it dragged some of the granules, what generated a descent of the biomass concentration in the reactor from 16.0 to 4.7 g VSS/L.

Therefore the reduction of the volumetric exchange causes the modification not only of the biomass concentration retained in the system but also of its physical properties.

3. Physical characteristic of the aerobic granular biomass:

Large biomass concentrations were obtained in the aerobic granular reactors, with values between 10 and 15 g VSS/L. Developed aerobic granules were characterised by their excellent settling properties, with values of sludge volumetric index around 30 mL/g TSS and densities up to 60 g VSS/$L_{granule}$ in the reactor treating the effluent generated in a fish canning industry.

Breakage episodes of the granules and losses of biomass, when it reached the withdrawal level of the reactor, were observed in both granular systems. Even though variations in the biomass concentration in the systems were produced, this did not cause the destabilization of the granules.

The coexistence between disperse and granular biomass was possible in the granular reactor treating the liquid fraction of swine slurry, but the balance is delicate since the granules remained retained in the matrix of disperse biomass.

So, granular biomass is easily formed in aerobic conditions in systems treating agri-food effluents. The applied loads and also the operational conditions are responsible of their different properties.

4. Post-Treatment of the liquid fraction of swine slurry in a CANON system

The nitrogen from the liquid fraction of the swine slurry, aerobically pre-treated in an aerobic granular reactor, was satisfactory removed in a CANON reactor operated at 20 °C. The use of air pulses favoured the control of the dissolved oxygen in the bulk liquid in order to keep the microaerobic conditions. A load of 0.60 kg N/(m^3 d) was applied and the removal efficiency of 75% was achieved when the nitrogen concentration was of 300 mg N-NH_4^+/L.

The presence of the slowly or non biodegradable fraction of organic matter did not affect the operation of the CANON system due to the development of the biomass in two different forms. One of them was composed by granules of 2.6 mm of diameter, enriched mainly in anammox bacteria, and the other by disperse biomass, enriched in ammonia oxidizing and heterotrophic bacteria. The development of these two forms minimised the competition between the ammonia oxidizing, anammox and heterotrophic bacteria for the common substrates.

Global results indicated that a combined system, composed by an aerobic granular reactor and a CANON system, was able to remove 2.12 kg COD/(m^3 d) and 0.30 kg N/(m^3 d) with removal efficiencies of 90% and 70%, respectively. Nevertheless nitrogen loads of 0.46 kg N/(m^3 d) were treated in a single CANON unit meaning that this is not the limiting process.

5. Characterisation of microbial populations:

The molecular biology techniques such as FISH, DGGE or cloning were useful tools for the identification of the main microbial populations involved in the organic matter and nitrogen removal in different types of reactors and also for visualise the filamentous bacteria present in the formed granules.

5.1. Involved in the organic matter removal

The main bacterial populations involved in the organic matter removal that are present in the granules obtained in aerobic conditions experienced changes during the time of operation of the systems. Identified bacterial populations are commonly present in activated sludge samples which means that a population specially dedicated to the granules formation was not detected.

Different members of the subclasses *Alpha*-, *Beta*- and *Gammaproteobacteria* and of the phylum *Bacteroidetes* and *Chloroflexi* were identified by means of cloning and DGGE in the reactor that treated the effluent from the fish canning industry. The cluster analysis of the DGGE bands obtained from biomass samples collected in different operational days indicated that bacteria related with the phylum *Bacteroidetes* and *Thauera* were present in the first stages of operation of the reactor, whereas *Thiothrix* and *Chloroflexi* developed at the end of the operation.

The use of specific FISH probes allowed to visualise in situ the majority of the populations of heterotrophic bacteria previously identified. Bacteria belonging to the phylum *Bacteroidetes*, the family *Comamonadaceae* and the genus *Zoogloea* and *Thauera* were observed. Besides, the different morphology of the *Bacteroidetes* bacteria in two different samples from the reactor was checked.

Because of the special characteristics of the feeding of the CANON system with presence of organic matter, heterotrophic bacteria were also developed, and they were identified by cloning as belonging to the phylum *Bacteroidetes*, and the families *Comamonadaceae* and *Rhodocyclaceae*.

5.2. Involved in the nitrogen removal

In these systems fed with high concentrations of ammonium, *Nitrosomonas* bacteria use to be the more abundant ammonia oxidizing bacteria.

The use of specific FISH probes allowed visualising in situ the populations of ammonia oxidizing bacteria *Nitrosomonas* responsible for the nitrogen oxidation in the reactor treating the effluent of the fish canning industry and in the CANON system. Due to the fact that it is a merely heterotrophic system, the amount of ammonia oxidizing bacteria in the granular system was around 8% whereas in the autotrophic CANON system it was of 35%.

The anammox bacteria responsible of the anaerobic ammonia oxidation in the CANON process were identified by means of cloning. These bacteria belonged to the species *C*. "Brocadia fulgida" and *C*. "Brocadia anammoxidans". By means of the FISH technique their abundance was estimated in 30%. The coexistence of different anammox species in a same system is not frequent and can be associated to the operational conditions of the reactor itself.

5.3. Presence of filamentous bacteria

Filamentous type organisms were observed in three aerobic granular reactors that treated the effluent from a fish canning industry, the effluent from a plant processing marine products and a synthetic effluent, respectively. The development of different species in different periods of operation was favoured because of the characteristics of each one of the systems. The study of previous operational periods indicated that their origin is the own treated wastewater or the inoculum used for the reactor start up.

In the reactor treating the wastewater from the fish canning industry *Thiothrix* and *Chloroflexi* bacteria were visualized. In the reactor treating the effluent from a plant processing marine products not only *Meganema perideroedes* bacteria but and also eukaryotic organisms of the Fungi kingdom were identified. *Chloroflexi* and *Sphaerotilus natans* bacteria were observed in the reactor fed with synthetic medium supplemented coagulant and floculant.

From the analysis of the operational conditions of each one of the systems it was postulated that the excessive development of filamentous organisms would be the responsible of the breakage of the aerobic granules.

SOME REFLECTIONS AND FUTURE PERSPECTIVES

It is necessary to underline that a unique option for wastewater treatment does not exist and that the factors that determine that a system is better than another are multiple. Aerobic granular systems will be suitable in those situations in which the aim is the organic matter removal and the nitrogen content reduction and where the space limitations require the implantation of more compact systems. The CANON system operated at low temperatures arises like a post-treatment of effluents with a small carbon/nitrogen ratio from which the reduction of the nitrogen content is necessary because of the current legislation.

At laboratory level to continue with the optimisation of the distribution of the operational cycles of the reactors is necessary, in order to achieve a higher removal efficiency in terms of nitrogen removal. This optimisation has to be done for each type of wastewater treated, since its composition is determinant in the operation of the reactor. Modifications in the oxygen concentration or the length of the cycle could influence the concentration profiles of each of the substrates.

Aerobic granular systems and the CANON process have demonstrated to be very efficient at laboratory scale regarding to organic matter and nitrogen removal of different wastewater of industrial origin, so its implementation at industrial scale is promising.

However different aspects that have to be considered before its implantation need to be optimized:

1. To establish recirculation systems of the used air in the aerobic systems to be able to minimise the aeration costs, since high flow rates are required to keep the biomass in suspension and to achieve the hydrodynamic stress that promotes the formation of granules.

2. To establish biomass purges to avoid events of withdrawal with the effluent due to the excessive growth of the granules, to control the solids retention time, and to attach a small settler to reduce the solids in the spilling point.

Although the use of specific techniques like FISH, DGGE or cloning for the identification of microbial populations seems to be of difficult application in wastewater treatment plants, the knowledge that they provide can be of great utility for the design and operation of biological systems. Therefore they can stop being considered like real black boxes in which only the inlets and outlets of the system are important.

List of acronyms and symbols

A	Adenine	——
amoA	ammonia monooxygenase subunit A	——
Anammox	ANaerobic AMMonium Oxidation	——
AOA	Ammonia Oxidizing Archaea	——
AOB	Ammonia Oxidizing Bacteria	——
AOR	Ammonium Oxidizing Rate	g N /L d
APHA	American Public Health Association	——
APS	Ammonium persulfate	——
AUSB	Aerobic Upflow Sludge Blanket	——
AWWA	American Water Works Association	——
BAS	Biofilm Airlift Suspension Reactor	——
BLAST	Basic Local Alignment Search Tool	——
C	Cytosine	——
CANON	Complete Autotrophic Nitrogen removal Over Nitrite	——
CLSM	Confocal Laser Scanning Microscope	——
COD	Chemical Oxygen Demand	g/L
COD/N	Chemical oxygen demand to nitrogen ratio	——
CSTR	Continuous Stirring Tank Reactor	——
Cy3	Cyanine 3	——
Cy5	Cyanine 5	——
d	Diameter	mm
DAPI	4',6-DiAmidino-2-Phenylindole	——
DGGE	Denaturing Gradient Gel Electrophoresis	——
DNA	Deoxyribo-Nucleic Acid	——
dNTPs	DeoxyriboNucleotide TriPhosphates	——
DO	Dissolved Oxygen concentration	mg O_2/L
EBPR	Enhanced Biological Phosphorus Removal	——
EDTA	Ethylene-Diamine-Tetra-Acetic acid	——
EMBL	European Molecular Biology Laboratory	——
EPS	Extracellular Polymeric Substances (exopolysaccharides)	——
F	Formamide	——
F/M	Food to microorganism ratio	——
FA	Free Ammonia	g N/L

FAOSTAT	Food and Agriculture Organization of the United Nations Statistical Database	——
FAS	Ferrous Ammonium Sulphate	——
FISH	Fluorescent *In Situ* Hybridization	——
FITC	Fluorescein IsoThioCyanate	——
FNA	Free Nitrous Acid	g N/L
G	Guanine	——
GSBR	Granular Sequencing Batch Reactor	——
H/D	Height to Diameter ratio	——
HAO	HydroxylAmine Oxidoreductase	——
HZO	HidraZine Oxidoreductase	——
HRT	Hydraulic Retention Time	d
IC	Inorganic Carbon	g/L
ISS	Inorganic Suspended Solids	g/L
IWA	International Water Association	——
MABR	Membrane Aerated Biofilm Reactor	——
MBR	Membrane Biological Reactor	——
N	Nitrogen	——
NCBI	National Center for Biotechnology Information	——
NLR	Nitrogen Loading Rate	kg N /(m^3 d)
NOB	Nitrite Oxidizing Bacteria	——
NOR	Nitrite OxidoReductase	——
NRR	Nitrogen Removal Rate	kg N /(m^3 d)
OLAND	Oxygen-Limited Autotrophic Nitrification-Denitrification	——
OLR	Organic Loading Rate	kg COD /(m^3 d)
PAO	Polyphosphate Accumulating Organisms	——
P	Phosphorous	——
PBS	Phosphate Buffer Solution	——
PHA	Poly-Hydroxy-Alkanoates	——
PHB	Poly-Hydroxy-Butyrate	——
PLC	Programmable Logic Controller	——
PCR	Polymerase Chain Reaction	——
RBC	Rotating Biofilm Contactor	——
rRNA	Ribosomal Ribo-Nucleic Acid	——
SBR	Sequencing Batch Reactor	——
SBRP	Air pulsing Sequencing Batch Reactor	——
SHARON	Single reactor system for High-activity Ammonia Removal over Nitrite	——
SEM	Scanning Electron Microscope	——

SNAP	Single-stage Nitrogen removal using Anammox and Partial nitritation	——
SRT	Solids Retention Time	d
SVI_n	Sludge Volume Index, after n minutes of settling	mL /g VSS
T	Thymine	——
TAE	Tris base, acetic acid and EDTA	——
TEMED	N,N,N',N'-TEtraMethylEneDiamine	——
TC	Total Carbon	g /L
TN	Total Nitrogen	g /L
TOC	Total Organic Carbon	g/L
Tris	Tris(hydroxymethyl)aminomethane	——
TSS	Total Suspended Solids	g/L
UASB	Upflow Anaerobic Sludge Blanket	——
UV	Ultraviolet	——
VER	Volumetric Exchange Ratio	%
VSS	Volatile Suspended Solids	g/L
WPCF	Water Pollution Control Facility	——
WWTPs	Wastewater Treatment Plants	——
Y	Yield coefficient	g SSV /g COD

Subindex

assimilated	Assimilated
denitrified	Denitrified
eff	Effluent
FAS	Ferrous ammonium sulphate
feret	Feret
granule	Granule
inf	Influent
mix	Mixture
max	Maximal
obs	Observed
r	Reactor
removed	Removed
s	Soluble
T	Total
treat	Treated

List of publications

International journal publications

Figueroa, M., Vázquez-Padín, J.R., Campos, J.L., Méndez R. and Mosquera-Corral A. (2006). Fluorescent in situ hybridization technique applied to the identification of bacteria from activated sludge samples. *Luminiscence*, 21: 383-385.

Figueroa, M., Mosquera-Corral, A., Campos, J.L. and Méndez R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. *Water Science and Technology*, 58: 479-485.

Arrojo, B., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez R. (2008). Influence of gas flow-induced shear stress on the operation of the Anammox process in a SBR. *Chemosphere*, 72: 1687- 1693.

Belmonte, M., Vázquez-Padín, J.R., **Figueroa, M.**, Franco, A., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2009). Characteristics of aerobic granules rich in autotrophic ammonium-oxidizing bacteria in a sequencing batch reactor. *Process Biochemistry*, 44: 602-606.

Vázquez-Padín, J.R., Fernández, I., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2009). Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature. *Bioresource Technology*, 100: 2988-2994.

Vázquez-Padín, J.R., Pozo, M.J., Jarpa, **Figueroa, M.**, M., Franco, A., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2009). Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR. *Journal of Hazardous Materials*, 166: 336-341.

Vázquez-Padín, J.R., **Figueroa, M.**, Fernández, I., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2009). Post-treatment of effluents from anaerobic digesters by the Anammox process. *Water Science and Technology*, 60: 1135-1143.

Vázquez-Padín, J.R., **Figueroa, M.**, Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2009). Population dynamics of nitrite oxidizers in nitrifying granules. *Water Science and Technology*, 60: 2529-2536.

Campos, J.L., **Figueroa, M.**, Mosquera-Corral, A. and Méndez, R. (2009). Aerobic sludge granulation: state-of-the-art. *International Journal of Environmental Engineering*, 1: 136-151.

Vázquez-Padín, J.R., **Figueroa, M.**, Campos, J.L., Mosquera-Corral, A. and Méndez R. (2010). Nitrifying granular systems: A suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology*, 74: 178-186.

Jungles, M. K., **Figueroa, M.**, Morales, N., Val del Río, A., Ribeiro da Costa R. H., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2011). Start up of a pilot scale aerobic granular reactor for organic matter and nitrogen removal. *Journal of Chemical Technology & Biotechnology,* 86: 763-768.

Figueroa, M., Val del Río, A., Campos, J.L., Mosquera-Corral, A. and Méndez R. (2011) Treatment of high loaded swine slurry in an aerobic granular reactor. *Water Science and Technology,* 63: 1808-1814.

Mosquera-Corral, A., Arrojo, B., **Figueroa, M.**, Campos J.L. and Méndez R. (2011) Aerobic Granulation in a Mechanical Stirred SBR: treatment of low organic loads. *Water Science and Technology,* 64*:* 155-161.

Val del Río A., **Figueroa, M.**, Arrojo B., Mosquera-Corral A., Campos J.L., García-Torriello G. and Méndez R. Aerobic granular SBR systems applied to the treatment of industrial effluents. *Journal of Environmental Management. Accepted.* doi:10.1016/j.jenvman.2011.03.019

Spanish journal publications

Arrojo, B., Vázquez-Padín, J.R., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. y Méndez, R. (2007). Reactores de biomasa granular: más capacidad en menos espacio. *Tecnología del Agua*, 287: 32-39.

Dapena, A., Fernández, I., **Figueroa, M.**, Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L. y Méndez, R. (2007). Proceso Anammox: Un cortocircuito en el Ciclo del Nitrógeno para la depuración de aguas residuales. *Revista Técnica de Medio Ambiente,* 116: 34-47.

Figueroa, M., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., Sanz J.L. y Méndez, R. (2008). Identificación de microorganismos implicados en la eliminación de nitrógeno en sistemas de tratamiento de aguas residuales mediante la técnica FISH. *Tecnología del Agua,* 300: 66-74.

Figueroa, M., Alonso-Gutiérrez, J., Campos,J.L., Méndez, R. y Mosquera-Corral, A. (2010). Identificación de microorganismos implicados en la eliminación de nitrógeno en sistemas de tratamiento de aguas residuales mediante técnicas de biología molecular. *Tecnología del Agua*, 317: 44-53

Figueroa, M., Val del Río, A., Morales, N., Mosquera-Corral, A., Méndez R. y Campos, J.L. (2011). Anammox: Hacia una eliminación sostenible del amonio en los efluentes de digestores anaerobios de purines. *Revista Técnica de Medio Ambiente*, 24: 84-90.

Figueroa, M., , Morales, N., Val del Río, A., Mosquera-Corral, A., Campos, J.L, y Méndez R. (2011). Tratamiento de la fracción líquida de purín de cerdo en reactores granulares aerobios SBR. InfoEnviro, 66: 108-111.

Book chapters

Méndez, R. Vázquez-Padín, J.R., Fernández, I. **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L., Larrea, L. and Colprim, J. (2008). Tratamiento de aguas de retorno a cabecera de planta en EDARs en *Tecnologías y estrategias para el rediseño de EDAR*. 53-69. Lápices 4

Campos, J.L., **Figueroa, M.**, Vázquez-Padín, J.R., Mosquera-Corral, A., Roca, E. and Méndez R. (2009). Evaluation of In-situ Sludge Reduction Technologies for Wastewater Treatment Plants en *Sludge: Types, Treatment Processes and Disposal*. Nova Science Publishers.

Figueroa, M., Val del Río, A., Morales, N., Campos, J.L., Mosquera-Corral, A. and Méndez R. (2009). Nitrogen removal in aerobic granular systems en *Environmental Technologies to Treat Nitrogen Pollution*. 373-401. IWA Publishing

Campos, J.L., Vázquez-Padín, J.R., **Figueroa, M.**, Fajardo C., Mosquera-Corral, A. and Méndez R. (2009). Novel biological nitrogen removal processes: Applications and perspectives en *Fluid waste disposal.* Nova Science Publishers.

Mosquera-Corral, A., **Figueroa, M.**, Morales, N., Val, A., Campos, J.L. and Méndez, R. (2009) Tecnologías basadas en biomasa granular aerobia en *Tecnologías Avanzadas para el Tratamiento de Aguas Residuales*. 48-68. Lápices 4

Mosquera-Corral, A., **Figueroa, M.**, Morales, N., Val, A., Campos, J.L. and Méndez, R. (2010) Aerobic granulation technology **en** *Innovative Technologies for Urban Wastewater Treatment Plants*. 35-58. Lápices 4

Figueroa, M., Campos, J.L., Mosquera-Corral, A., Méndez, R. and Hung, Y.T. Aerobic granulation process for waste treatment en *Handbook of Environmental and Waste Management.* World Scientific Publishing. *Corrected proofs.*

Campos, J.L., **Figueroa, M.**, Morales, N., Fajardo, C., Vázquez-Padín, J.R., Mosquera-Corral, A., and R. Méndez. Advanced systems for nitrogen removal from effluents produced in the fish canning industry en *Meat, Poultry and Seafood Industries*. Nova Science Publishers. *Accepted*

Conference Proceedings

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. (2005). Start-up of Anammox process: different reactor alternatives. IWA Specialized Conference Nutrient Management in Wastewater Treatment Processes and Recycle Streams. (Krakow, Polonia).

Vázquez-Padín, J.R., Arrojo, B., Mosquera-Corral, A., **Figueroa, M.**, Campos, J.L. and Méndez, R. (2005). Granulation sludge for aerobic wastewater treatment: nitrogen removal. X Congreso Mediterráneo de Ingeniería Química. (Barcelona, España).

Vázquez-Padín, J.R., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2006). Nitrifying granules applied for nitrite accumulation. International Conference on Environmental Biotechnology (ISEB ESEB JSEB). (Leipzig, Alemania).

Figueroa, M., Vázquez-Padín, J.R., Campos, J.L., Méndez, R. and Mosquera-Corral, A. (2006). Fluorescent in situ hybridization technique applied to the identification of bacteria from activated sludge samples. XIIth International Symposium on Luminiscence Spectrometry-Detection Techniques in Biomedical, Environmental and Food Analysis. (Lugo, España).

Vázquez-Padín, J.R., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2006). Operation of nitrifying granules in SBR. International Water Conference (IWC'2006). (Porto, Portugal).

Vázquez-Padín, J.R., **Figueroa, M.**, B. Arrojo, Mosquera-Corral, A., Campos, J.L. and R. Mendez. (2006). Why do nitrifying granules accumulate nitrite?. 2nd Workshop: "Aerobic Granular Sludge". (Delft, Holanda).

Vázquez-Padín, J.R., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2006). Respirometry: an useful tool for the design and operation of wastewater treatment plants. I Congreso del Grupo Especializado de Microbiología Industrial y Biotecnología Microbiana. (A Coruña, España).

Figueroa, M., Vázquez-Padín, J.R., Arrojo, B., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2006). Aerobic granules for the integrated wastewater treatment. I Congreso del Grupo Especializado de Microbiología Industrial y Biotecnología Microbiana. (A Coruña, España).

Vázquez-Padín, J.R., **Figueroa, M.**, B. Arrojo, Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2007) Nitrification in aerobic granular sludge limited by DO diffusion.CLONIC Final Workshop. (Barcelona, España).

Fernández, I., Dosta, J., Vázquez-Padín, J.R., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2008). Operation of an Anammox SBR at low temperatures. 4th Sequencing Batch Reactor Conference (Roma, Italia).

Vázquez-Padín, J.R., Pozo, M.J., Jarpa, M., **Figueroa, M.**, Franco, A., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2008) Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsating SBR. 2nd International Conference on Engineering for Waste Valorisation (Patras, Grecia).

Vázquez-Padín J.R., **Figueroa, M.**, Mosquera-Corral A., Campos J.L. and Méndez R. (2008). From partial nitrification to CANON at room temperature in an aerobic granular SBR. Third International Meeting on Environmental Biotechnology and Engineering (Mallorca, España).

Vázquez-Padín, J.R. **Figueroa, M.**, Fernández, I., Mosquera-Corral, A., Campos, J.L. and Méndez R. (2008). Post-treatment of effluents from anaerobic digesters by the Anammox process. IX taller y simposio latinoamericano de digestión anaerobia (Isla de Pascua, Chile).

Figueroa, M., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2008). Treatment of saline wastewater in SBR aerobic granular reactors. 4th Sequencing Batch Reactor Conference (Roma, Italia).

Figueroa, M., B. Arrojo, Mosquera-Corral, A., Campos, J.L. and Méndez, R.. (2008). Tratamiento de purines en reatores granulares aerobios SBR. Congreso: I Congreso Español de Gestión Integral de Deyecciones Ganaderas (Barcelona, España).

Figueroa, M., van der Biezen, E., Kartal,B., Mosquera-Corral, A., Campos, J.L., Méndez, R. and Jetten, M.S.M. (2008). Operation and identification of microorganisms involved in CANON reactor. The Nijmegen N-cycle Meeting (Nijmegen, Holanda).

Val del Río, A., **Figueroa, M.**, Arrojo, B., Mosquera-Corral, A., Campos, J.L., García-Torriello, G. and Méndez, R. (2008) Aerobic granular SBR systems applied to the treatment of industrial effluents. Third International Meeting on Environmental Biotechnology and Engineering. (Palma de Mallorca, España).

Morales, N., **Figueroa, M.**, Val del Río, A., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2008). Start up and operation of pilot scale aerobic granular SBR. Third International Meeting on Environmental Biotechnology and Engineering. (Palma de Mallorca, España).

Figueroa, M., Vázquez-Padín, J.R., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2009). Identification of bacterial populations in an aerobic granular SBR performing the partial nitrification and CANON processes. Conference on microbial population dynamics in biological wastewater treatment (Aalborg, Dinamarca).

Vázquez-Padín, J.R., **Figueroa, M.**, Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2009). Population dynamics of nitrite oxidizers in nitrifying granules. IWA second specialized conference on nutrient management in wastewater treatment processes (Krakow, Polonia).

Rodríguez-Díaz, M., van Alen, T., **Figueroa, M.**, Ettwig, K.F. and Strous, M. (2009) Screening for denitrifying methanotrophic bacteria of the NC10 phylum in environmental samples. FEMS 2009: 3rd Congress of European Microbiologist. (Gothenburg, Suecia)

Figueroa, M., Val del Río, A., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2010). Treatment of high loaded swine slurry in an aerobic granular reactor. Sustainable Solutions for Small Water and Wastewater Treatment Systems S2Small 2010. (Girona, España).

Jungles, M.K., **Figueroa, M.**, Morales, N., Vázquez-Padín, J.R., Costa, R.H.R., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2010) Start up of a pilot scale aerobic granular reactor. Sustainable Solutions for Small Water and Wastewater Treatment Systems S2Small 2010. (Girona, España).

Morales, N., **Figueroa, M.**, Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2010) Aerobic Granular Biomass in a Continuous Stirred Tank Reactor. Sustainable Solutions for Small Water and Wastewater Treatment Systems S2Small 2010. (Girona, España).

Campos, J.L., **Figueroa, M.**, Fernández, I., Mosquera-Corral, A. and Méndez, R. (2010) Anammox: ¿El proceso del futuro para la eliminación de amonio de los efluentes de los digestores de purines?. II Congreso Español de Gestión Integral de Deyecciones Ganaderas. (Barcelona, España)

Vázquez-Padín, J. R., **Figueroa, M.**, Morales, N., Campos, J.L., Mosquera-Corral, A. and Méndez, R. (2010) Autotrophic nitrogen removal in granular reactors. Conferencia Nacional de Jóvenes Profesionales del Agua de España (Spain National Young Water Professionals Conference, Spain NYWPC) (Barcelona, España)

Mosquera-Corral, A., Arrojo, B., **Figueroa, M.**, Campos, J.L. and Méndez, R. (2010) Aerobic Granulation in a Continuous Stirred SBR treating low organic loads. IWA World Water Congress and Exhibition (Montreal, Canadá)

Méndez, R., **Figueroa, M.**, Vázquez-Padín, J.R., Mosquera-Corral, A. and Campos, J.L. (2010). Advanced biotechnologies for the removal of nutrients. 5th International Meeting on Biotechnology. (Pamplona, España).

Mery C., Guerrero L., Alonso-Gutiérrez J., **Figueroa, M.**, Lema J., Montalvo S. and Vidal G. (2010) Microorganism in batch reactors related to nitrogen removal using different sizes of natural zeolite. 12th World Congress on Anaerobic Digestion. (Guadalajara, México)

Mery C., Guerrero L., Alonso-Gutiérrez J., **Figueroa, M.**, Lema J., Montalvo S. and Vidal G. (2010). Aplicación de técnicas de biología molecular para la identificación de microorganismos nitrificantes y desnitrificantes adheridos a zeolitas naturales. XXXII Congreso Interamericano de Ingenieria Sanitaria y Ambiental AIDIS. (Punta Cana, Rep. Dominicana).

Belmonte, M., Vázquez-Padín, J.R., **Figueroa, M.**, Campos J. L., Mendez, R., Mosquera-Corral, A. and Vidal, G. (2011). Producción de óxido nitroso en ensayos desnitrificantes vía nitrito utilizando efluente de purín de cerdo. II Simposio Internacional sobre Gerenciamento de Resíduos Agropecuários e Agroindustriais, SIGERA II, (Foz do Iguaçu, Brasil).