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# Aerobic granular biomass systems: a challenge for a better wastewater treatment and sludge management

Memoria presentada por

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Para optar ó grao de Doutor pola

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# Resumen

El desarrollo de la sociedad y la actividad humana (agricultura, industria, negocios, hogares, etc.) lleva asociado consigo un aumento de la contaminación, siendo uno de los efectos más importantes la producción de agua residual. La descarga del agua residual sin tratar en los cuerpos de agua puede provocar la desaparición de determinadas formas de vida, la pérdida de biodiversidad, la distorsión de los sistemas acuáticos, un exceso de fertilización y afectar a las fuentes de agua potable provocando problemas en la salud pública. Por tanto, para reducir estos problemas y garantizar una adecuada disposición en el medio ambiente, es necesario tratar el agua residual. Sin embargo a consecuencia de este tratamiento se genera otro tipo de residuo que son los “lodos” y que también requieren de tratamiento.

El tratamiento del agua residual mediante procesos biológicos se lleva a cabo normalmente en sistemas convencionales de lodos activos, los cuales requieren de grandes superficies para su implantación y la presencia de unidades de separación (decantadores secundarios) debido a las pobres propiedades de sedimentación del lodo. Los sistemas basados en la biomasa granular aerobia son una buena alternativa a estos sistemas convencionales debido a la menor necesidad de terreno para su implantación. Esto es debido al diseño del reactor (con una relación altura/diámetro grande) y a las buenas propiedades de sedimentación de la biomasa que hacen innecesario el uso de decantadores secundarios. La gran concentración de biomasa alcanzada en estos sistemas hace posible poder tratar grandes cargas de materia orgánica y nitrógeno, lo cual también contribuye a reducir el volumen de reactor necesario. Por otro lado la agregación de la biomasa en gránulos permite que se desarrollen diferentes condiciones (aerobias, anóxicas y anaerobias). Esto da lugar a la proliferación de diversas poblaciones microbianas capaces de llevar a cabo diferentes procesos biológicos. Esto significa que en una única unidad es posible llevar a cabo de forma simultánea la eliminación de carbono, nitrógeno y fósforo. Otra ventaja de los sistemas granulares respecto de los lodos activos es su menor producción de lodos. Esto es importante ya que la gestión de los lodos producidos como consecuencia del proceso de tratamiento del agua residual supone más del 50% de los costes operacionales de las Estaciones Depuradoras de Aguas Residuales (EDARs). Además las regulaciones europeas respecto a la gestión de estos lodos se están volviendo cada vez más estrictas. Por lo que hoy en día se están desarrollando nuevas tecnologías que permitan, primero, reducir la cantidad de lodos producidos y, segundo, mejorar su posterior tratamiento. En este sentido los sistemas basados en biomasa granular aerobia son una de las tecnologías propuestas para sustituir los sistemas convencionales de lodos activos debido a su configuración compacta.

Los primeros trabajos publicados sobre la formación de biomasa granular aerobia aparecieron en la década de los 90. Desde entonces las investigaciones se han centrado en la mejora de los diferentes aspectos relacionados con el escalado de esta tecnología. Se sabe que el proceso de granulación puede estar afectado por numerosos factores, siendo uno de ellos el tipo de substrato. Hasta ahora los resultados de diversos trabajos parecen indicar que es posible formar gránulos aerobios tratando diferentes tipos de substratos, pero también que la diversidad microbiana y la estructura de los gránulos aerobios están estrechamente relacionadas con el tipo de substrato empleado. El desarrollo de biomasa granular aerobia ha sido estudiado tratando diferentes tipos de medios sintéticos y aguas industriales, lo cual indica que es posible hacer crecer gránulos aerobios con substratos complejos. Esto es de interés para las industrias, ya que normalmente la disponibilidad de suelo industrial para la implantación de la EDAR está limitada. Esta limitación es más evidente en el caso de aquellas industrias situadas cerca de la costa, que están normalmente dedicadas al procesado de productos del mar (conserveras, plantas de acuicultura, etc.). En este sentido en el Capítulo 3 se ha estudiado el desarrollo de biomasa granular aerobia con el efluente procedente de este tipo de industria, con especial atención en las características de los gránulos aerobios formados y en las eficacias de eliminación de materia orgánica y nitrógeno. También ha sido estudiada la estabilidad de la biomasa granular aerobia frente a cambios bruscos en la carga aplicada, ya que el agua residual generada en las industrias de productos del mar está caracterizada por una gran variabilidad en su composición.

Además de estudiar la posibilidad de poder aplicar los sistemas de biomasa granular aerobia con diferentes tipos de aguas residuales, también es importante conocer el efecto que determinados compuestos específicos pueden tener en el desarrollo de los gránulos aerobios. En este sentido numerosos trabajos ya publicados han estudiado los efectos de compuestos como fenol, piridina, metales pesados y tintes. Sin embargo el efecto de la presencia de reactivos como los coagulantes y floculantes aun no se ha determinado, a pesar de que son muy comúnmente empleados como pre-tratamiento para la eliminación de sólidos antes del proceso biológico. El agua residual empleada en el Capítulo 3 fue recogida en la propia industria después de la unidad de pre-tratamiento donde coagulante y floculante eran añadidos, y los resultados obtenidos parecen indicar que la concentración residual de estos compuestos podría afectar las propiedades de la biomasa granular aerobia. Por esta razón en el Capítulo 4 se ha estudiado el efecto de estos compuestos usando como agua residual un medio sintético y así evitar la interferencia de otras substancias complejas, que pueden estar presentes en el efluente industrial procedente de la planta de procesado de productos del mar.

Los sistemas granulares aerobios son operados en reactores secuenciales discontinuos con una fase de alimentación inicial corta seguida de una fase de reacción que permite someter la biomasa a un régimen de saciedad-hambruna, donde el periodo de saciedad es corto y el de hambruna largo. Estas condiciones son las adecuadas para seleccionar los microorganismos apropiados para obtener gránulos densos y compactos. Sin embargo un periodo de hambruna excesivamente largo puede no ser necesario y llevar asociado consigo un consumo extra de energía y una pérdida en la capacidad de tratamiento. Además la eliminación de nitrógeno está

limitada por el proceso de desnitrificación debido a las altas concentraciones de oxígeno disuelto durante el período de hambruna. Por eso en el Capítulo 5 se analizaron otro tipo de configuraciones del ciclo con el objetivo de determinar la longitud y la distribución del ciclo de operación que permita maximizar la capacidad de tratamiento y la eliminación de nitrógeno, respectivamente.

Aunque la producción de lodos con los sistemas de biomasa granular aerobia sea menor que con los sistemas convencionales de lodos activos, su posterior tratamiento sigue siendo aún necesario. Sin embargo hoy en día los trabajos de investigación sobre su posible tratamiento son prácticamente nulos, debido a que los sistemas de biomasa granular aerobia están aún en desarrollo y bajo estudio a escala piloto. Por eso es de gran interés el estudio de las posibles vías de tratamiento de este tipo de lodo, para poder escalar estos sistemas y aplicarlos a la industria. La digestión anaerobia es ampliamente utilizada para el tratamiento de los lodos generados en las EDARs debido a que permite estabilizar su contenido en materia orgánica y reducir la cantidad de sólidos, así como producir biogás que puede ser recuperado en la misma instalación como fuente de energía. Sin embargo la degradación anaerobia del lodo producido en los sistemas convencionales de lodos activos está normalmente limitada por la etapa de hidrólisis y con porcentajes entorno al 30-50%. Para mejorar la digestión anaerobia de los lodos activos se puede aplicar un pre-tratamiento (térmico, mecánico, químico o una combinación de ellos), que permita la destrucción de la matriz del lodo (flóculos) para mejorar la hidrólisis. Debido a que el lodo generado por los sistemas de biomasa granular aerobia tiene una naturaleza similar a la de los lodos activos (procedente de un tratamiento biológico) el uso de la digestión anaerobia puede también ser una solución interesante para tratarlo, así como la aplicación de un pre-tratamiento antes del proceso anaerobio, que podría contribuir a mitigar el posible efecto del estado de agregación del lodo en gránulos. Por eso en los Capítulos 6 y 7 el objetivo propuesto fue estudiar la posibilidad de aplicar la digestión anaerobia para el tratamiento del lodo granular aerobio, primero mediante experimentos en discontinuo (Capítulo 6) y luego con la operación de un reactor continuo anaerobio a escala laboratorio (Capítulo 7). En ambos capítulos también se estudió la aplicación de un pre-tratamiento térmico para mejorar la digestión anaerobia del lodo granular.

Dentro de este contexto la presente tesis está enmarcada en el campo del tratamiento de aguas residuales con la aplicación de la tecnología basada en biomasa granular aerobia (Capítulos 3, 4 y 5) y en el posterior tratamiento del lodo granular generado mediante digestión anaerobia (Capítulos 6 y 7). Los contenidos principales de cada uno de los capítulos así como los objetivos alcanzados se detallan a continuación:

En el Capítulo 1 se ofrece una visión general sobre los procesos de tratamiento de las aguas residuales, centrada en el sistema convencional de lodos activos y en los inconvenientes asociados a la aplicación de esta vieja tecnología (gran superficie para su implementación, baja capacidad de tratamiento y grandes cantidades de lodo generadas con malas propiedades de sedimentación). Los sistemas de biomasa granular aerobia se presentan como una posible alternativa para resolver parte de estos problemas. También se realiza una revisión acerca de los factores que afectan la formación de biomasa granular aerobia así como de sus

aplicaciones. Finalmente se describen los aspectos básicos de la digestión anaerobia de lodos con especial atención en la microbiología del proceso y en los posibles métodos de pre-tratamiento.

En el Capítulo 2 se describen los materiales y métodos empleados en la tesis. Estos engloban el análisis de las fases líquida, gas y sólida. Algunos parámetros convencionales como el pH, oxígeno disuelto, Demanda Química de Oxígeno (DQO), composición del biogás o concentración de sólidos se midieron de acuerdo al Standard Methods. Otros análisis se realizaron después de adaptarlos a la investigación de esta tesis, como la caracterización de la biomasa granular aerobia mediante el Índice Volumétrico de Lodos (IVL), la determinación de la densidad de los gránulos, la aplicación del análisis digital de imagen para determinar la morfología y el diámetro medio de los gránulos y la medida de la concentración de Poly-Hydroxy-Alcanoatos (PHA). También se describe la aplicación de la técnica de Hibridación *in situ* con Fluorescencia (FISH) para la identificación de las poblaciones microbianas involucradas en los procesos biológicos, especialmente en la digestión anaerobia.

En el Capítulo 3 se estudió el arranque y operación de un reactor secuencial con biomasa granular aerobia para el tratamiento de un efluente industrial procedente de una planta de procesado de productos del mar. Este efluente industrial estaba caracterizado por una composición muy variable debido a los diferentes productos procesados en la planta (calamares, langostinos, merluza, etc.). Además este efluente procedía de un tratamiento físico-químico donde se añadía coagulante y floculante. La operación del reactor granular aerobio estuvo dividida en dos etapas. En la primera (días 0-295) se promocionó el desarrollo de los gránulos aerobios mediante la aplicación de cargas orgánicas entre 2 y 4 kg $DQO_S/m^3 \cdot d$. A partir del día 130 de operación tuvo lugar el proceso de granulación y la biomasa granular aerobia desarrollada presentó buenas propiedades de sedimentación, con valores del IVL de 35 mL/g SST y de densidad de 60 g $SSV/L_{gránulo}$ entorno al día 170 de operación. El diámetro medio de los gránulos hasta este momento de operación osciló entre 2-3 mm, pero luego aumentó hasta valores medios de 11.0 mm en pocos días y posteriormente tuvo lugar la desintegración de los gránulos, probablemente debido a la presencia de mayores concentraciones residuales de coagulante y floculante. La eficacia de eliminación de la materia orgánica fue del 90% y, aunque las condiciones para la eliminación de nitrógeno no fueron optimizadas se alcanzaron porcentajes de eliminación del 65% y 30% de amonio y nitrógeno total, respectivamente. En la segunda etapa (días 296-330) se estudió la estabilidad de la biomasa granular aerobia frente a cargas orgánicas variables (desde 3 hasta 13 kg $DQO_S/m^3 \cdot d$) para aproximar la operación del reactor a las condiciones reales de la industria. Con este test de estabilidad frente a cargas variables la eficacia de eliminación de materia orgánica no se vio alterada pero las propiedades físicas de los gránulos y la eficacia de eliminación de nitrógeno empeoraron.

En el Capítulo 4 se operaron dos reactores secuenciales discontinuos para la obtención de biomasa granular aerobia con el objetivo de conocer el efecto del coagulante y floculante en la formación y características de los gránulos aerobios, puesto que los resultados obtenidos en el Capítulo 3 parecen indicar que estos compuestos, que son frecuentemente usados en el

tratamiento primario, pueden afectarlas. Ambos reactores fueron alimentados con un medio sintético y uno de ellos además fue suplementado con una cantidad residual de coagulante-floculante (CF), mientras que el otro sirvió de control. El principal efecto observado fue una peor capacidad de retención de la biomasa en el reactor alimentado con CF comparado con el control, lo cual implicó una menor concentración de sólidos (4.5 vs. 7.9 g SSV/L) y un mayor IVL (70 vs. 40 mL/g SST). El diámetro medio de los gránulos aerobios en el reactor CF fue mayor que en el control (5.0 vs. 2.3 mm). Además en el reactor CF la velocidad máxima de consumo de oxígeno disminuyó respecto al control pero las eficacias de eliminación de materia orgánica (90%) y nitrógeno (60%) no se vieron afectadas.

En el Capítulo 5 se cambió la distribución del ciclo operacional del reactor secuencial discontinuo con biomasa granular aerobia respecto a la distribución usada en los Capítulos 3 y 4, para mejorar la capacidad de tratamiento y la eliminación de nitrógeno. En un primer experimento se empleó el reactor control del Capítulo 4 con biomasa granular aerobia madura y se sometió a una disminución progresiva de la relación hambruna/saciedad disminuyendo la longitud de la fase aerobia. El propósito de este cambio de ciclo fue aumentar la capacidad de tratamiento del sistema. Los resultados obtenidos mostraron que fue posible reducir la relación hambruna/saciedad desde 10 hasta 5, consiguiendo un aumento de las cargas orgánica y nitrogenada tratadas en el sistema entorno al 33%. Las eficacias de eliminación de materia orgánica (97%) y de nitrógeno (64%) no se vieron afectadas mientras que se produjo un ligero empeoramiento de las características de los gránulos. En un segundo experimento la biomasa granular aerobia madura fue recogida de una planta piloto (100 L) que trataba purín de cerdo y usada para inocular dos reactores secuenciales discontinuos a escala laboratorio. En estos reactores se implementó una fase anóxica previa a la aerobia para mejorar la eficacia de eliminación de nitrógeno respecto a la operación de la planta piloto (con solo fase aerobia). Los resultados obtenidos mostraron que añadiendo la alimentación de una sola vez y con una fase anóxica de 30 minutos antes de la aerobia aumentaba la eficacia de eliminación de nitrógeno del purín de cerdo desde el 20% hasta el 60%. Sin embargo la configuración del ciclo con la alimentación continua y simultánea a la fase anóxica de 60 minutos, no solo no mejoraba la eficacia de eliminación de nitrógeno, sino que incluso empeoraba la de oxidación de amonio.

En el Capítulo 6 se estudiaron los efectos del pre-tratamiento térmico en las propiedades macroscópicas y bioquímicas del lodo granular aerobio, así como la mejora en la biodegradabilidad anaerobia después de la aplicación de este pre-tratamiento. La caracterización bioquímica de las muestras y los resultados obtenidos de los ensayos anaerobios en discontinuo también fueron utilizados para validar un modelo que permita estimar la biodegradabilidad anaerobia de la biomasa granular aerobia. Para conseguir estos objetivos se utilizaron dos lodos granulares aerobios procedentes de dos plantas piloto (100 L): un lodo granular de un reactor alimentado con purín de cerdo (G1) y otro procedente de un reactor alimentado con un medio sintético para simular un agua residual urbana (G2). Las temperaturas del pre-tratamiento utilizadas variaron entre 60-210 ºC para G1 y 170-210 ºC para G2, este pre-tratamiento se aplicó durante 20 minutos. Los resultados obtenidos para el porcentaje de biodegradabilidad anaerobio fueron del 33% para G1 y del 49% para G2, que son valores

similares a los referenciados para lodos activos (30-50%). El pre-tratamiento térmico antes de la digestión anaerobia mejoró la biodegradabilidad del lodo G1 respecto al mismo lodo sin pre-tratamiento para todas las temperaturas ensayadas: 21% a 60 ºC, 42% a 90 ºC, 64% a 115 ºC, 82% a 140 ºC, 88% a 170 ºC, 70% a 190 ºC y 58% a 210 ºC. Para el lodo G2 el pre-tratamiento térmico no mejoró la biodegradabilidad a 170 ºC y solo se consiguió una mejora del 14% y 18% a 190 y 210 ºC, respectivamente.

En el Capítulo 7 se estudió la viabilidad de aplicar la digestión anaerobia en continuo para el lodo granular aerobio. En este caso el lodo granular empleado fue recogido de la misma planta piloto que el lodo G1 en el Capítulo 6. El digestor anaerobio empleado tenía un volumen útil de 5 L y fue operado en condiciones mesofílicas (35 ºC) en tres etapas diferentes. La primera correspondió con la digestión anaerobia del lodo granular aerobio sin ningún tipo de pre-tratamiento, obteniéndose valores de biodegradabilidad anaerobia del 44% y de reducción de sólidos del 32%, los cuales se encuentran dentro del rango de los referenciados para lodos activos. La biodegradabilidad anaerobia en este experimento fue mayor que la obtenida en los ensayos en discontinuo del Capítulo 6 (33%), probablemente debido a cambios en la muestra del lodo granular aerobio, ya que desde que las muestras fueron tomadas de la planta piloto entre un experimento y otro transcurrió un año. La segunda etapa correspondió con la digestión anaerobia del lodo granular pre-tratado térmicamente. En este caso el pre-tratamiento térmico fue llevado a cabo a 130 ºC durante 20 minutos. La temperatura de pre-tratamiento fue escogida de acuerdo a los resultados obtenidos previamente en los ensayos en discontinuo (Capítulo 6). Este pre-tratamiento térmico mejoró el rendimiento del reactor un 32% y 47% en términos de biodegradabilidad y reducción de sólidos, respectivamente. En la tercera etapa el digestor anaerobio fue alimentado con una mezcla de lodo granular aerobio pre-tratado térmicamente y lodo primario sin tratar, puesto que la bibliografía indica que el pre-tratamiento del primario no es necesario. Esta mezcla produjo una mejora del 17% en la eliminación de sólidos respecto al lodo granular pre-tratado sólo, mientras que la biodegradabilidad disminuyó desde el 58% hasta el 53%, lo cual fue debido a que la relación $DQO_T$/SV de la mezcla respecto a sólo lodo granular pre-tratado era mayor. En este capítulo también se realizó un análisis de las poblaciones microbianas dentro del reactor anaerobio mediante la técnica FISH. Los principales hechos observados fueron que el dominio *Arquea* tuvo una mayor presencia que el dominio *Bacteria*. Además la abundancia de ambos dominios disminuyó con el cambio en la alimentación desde lodo granular sin tratar, hasta lodo granular pre-tratado y su mezcla con el lodo primario.

# Resumo

O desenvolvemento da sociedade e a actividade humana (agricultura, industria, negocios, fogares, etc.) leva asociado consigo un aumento da contaminación, sendo un dos efectos máis importantes a produción de auga residual. A descarga da auga residual sen tratar nos corpos de auga pode provocar a desaparición de determinadas formas de vida, a perda de biodiversidade, a distorsión dos sistemas acuáticos, un exceso de fertilización e afectar ás fontes de auga potable provocando problemas na saúde pública. Polo tanto, para reducir estes problemas e garantir unha axeitada disposición no medio, é necesario tratar a auga residual. Non obstante como consecuencia deste tratamento xérase outro tipo de residuo que son as "lamas" e que tamén requiren de tratamento.

O tratamento da auga residual mediante procesos biolóxicos lévase a cabo normalmente en sistemas convencionais de lamas activas, os cales requiren de grandes superficies para a súa implantación e a presenza unidades de separación (decantadores secundarios) debido ás pobres propiedades de sedimentación da lama. Os sistemas baseados na biomasa granular aerobia son unha alternativa a estes sistemas convencionais debido á menor necesidade de terreo para a súa implantación. Isto é debido ao deseño do reactor (cunha relación altura/diámetro grande) e ás boas propiedades de sedimentación da biomasa que fan innecesario o uso de decantadores secundarios. A gran concentración de biomasa acadada nestes sistemas fai posible poder tratar grandes cargas de materia orgánica e nitróxeno, que tamén contribúe a reducir o volume de reactor necesario. Por outro lado a agregación da biomasa en gránulos permite que se desenvolvan diferentes condicións (aerobias, anóxicas e anaerobias). Isto dá lugar á proliferación de diversas poboacións microbianas capaces de levar a cabo diferentes procesos biolóxicos. O cal significa que nunha única unidade é posible levar a cabo de forma simultánea a eliminación de carbono, nitróxeno e fósforo. Outra vantaxe dos sistemas granulares respecto das lamas activas é a súa menor produción de biomasa. Isto é importante xa que a xestión das lamas producidas como consecuencia do proceso de tratamento da auga residual supón máis do 50% dos custos operacionais das Estacións Depuradoras de Augas Residuais (EDARs). Ademais as regulacións europeas respecto á xestión destas lamas estanse a volver cada vez máis estritas. Polo que hoxe en día se están a desenvolver novas tecnoloxías que permitan, primeiro, reducir a cantidade de lamas producidas e, segundo, mellorar o seu posterior tratamento. Neste sentido os sistemas baseados en biomasa granular aerobia son unha das tecnoloxías propostas para substituír os sistemas convencionais de lamas activas debido á súa configuración compacta.

Os primeiros traballos publicados sobre a formación de biomasa granular aerobia apareceron na década dos 90. Dende entón as investigacións centráronse na mellora dos diferentes aspectos relacionados co escalado desta tecnoloxía. Sábese que o proceso de granulación pode estar afectado por numerosos factores, sendo un deles o tipo de substrato. Ata agora os resultados de diversos traballos parecen indicar que é posible formar gránulos aerobios tratando diferentes tipos de substratos, pero tamén que a diversidade microbiana e a estrutura dos gránulos aerobios están estreitamente relacionadas co tipo de substrato empregado. O desenvolvemento de biomasa granular aerobia foi estudado tratando diferentes tipos de medios sintéticos e augas industriais, o cal indica que é posible facer crecer gránulos aerobios con substratos complexos. Isto é de interese para as industrias, xa que normalmente a dispoñibilidade de chan industrial para a implantación da EDAR está limitada. Esta limitación é máis evidente no caso daquelas industrias situadas preto da costa, que están normalmente dedicadas ao procesado de produtos do mar (conserveiras, plantas de acuicultura, etc.). Neste sentido no Capítulo 3 estudouse o desenvolvemento de biomasa granular aerobia co efluente procedente deste tipo de industria, con especial atención nas características dos gránulos aerobios formados e nas eficacias de eliminación de materia orgánica e nitróxeno. Tamén foi estudada a estabilidade da biomasa granular aerobia fronte a cambios bruscos na carga aplicada, xa que a auga residual xerada nas industrias de produtos do mar está caracterizada por unha gran variabilidade na súa composición.

Ademais de estudar a posibilidade de poder aplicar os sistemas de biomasa granular aerobia con diferentes tipos de augas residuais, tamén é importante coñecer o efecto que determinados compostos específicos poden ter no desenvolvemento dos gránulos aerobios. Neste sentido numerosos traballos xa publicados estudaron os efectos de compostos como fenol, piridina, metais pesados e tinguiduras. Non obstante o efecto da presenza de reactivos como os coagulantes e floculantes aínda non se determinou, a pesar de que son moi comunmente empregados como pre-tratamento para a eliminación de sólidos antes do proceso biolóxico. A auga residual empregada no Capítulo 3 foi recollida na propia industria despois da unidade de pre-tratamento onde coagulante e floculante eran engadidos, e os resultados obtidos parecen indicar que a concentración residual destes compostos podería afectar ás propiedades da biomasa granular aerobia. Por esta razón no Capítulo 4 estudouse o efecto destes compostos usando como auga residual un medio sintético e así evitar a interferencia doutras substancias complexas, que poden estar presentes no efluente industrial procedente da planta de procesado de produtos do mar.

Os sistemas granulares aerobios son operados en reactores secuenciais descontinuos cunha fase de alimentación inicial curta seguida dunha fase de reacción que permite someter a biomasa a un réxime de saciedade-fame, onde o período de saciedade é curto e o de fame longo. Estas condicións son as adecuadas para seleccionar os microorganismos apropiados para obter gránulos densos e compactos. Non obstante un período de fame excesivamente longo pode non ser necesario e levar asociado consigo un consumo extra de enerxía e unha perda na capacidade de tratamento. Ademais a eliminación de nitróxeno está limitada polo proceso de desnitrificación debido ás altas concentracións de osíxeno disolto durante o período

de fame. Por iso no Capítulo 5 analizáronse outro tipo de configuracións do ciclo co obxectivo de determinar a lonxitude e a distribución do ciclo de operación que permita maximizar a capacidade de tratamento e a eliminación de nitróxeno, respectivamente.

Aínda que a produción de lamas cos sistemas de biomasa granular aerobia sexa menor que cos sistemas convencionais de lamas activas, o seu posterior tratamento segue sendo aínda necesario. Non obstante hoxe en día os traballos de investigación sobre o seu posible tratamento son practicamente nulos, debido a que os sistemas de biomasa granular aerobia están aínda en desenvolvemento e baixo estudo a escala piloto. Por iso é de grande interese o estudo das posibles vías de tratamento deste tipo de lama, para poder escalar estes sistemas e aplicalos á industria. A dixestión anaerobia é utilizada amplamente para o tratamento das lamas xeradas nas EDARs debido a que permite estabilizar o seu contido en materia orgánica e reducir a cantidade de sólidos, así como producir biogás que pode ser recuperado na mesma instalación como fonte de enerxía. Non obstante a degradación anaerobia da lama producida nos sistemas convencionais de lamas activas está normalmente limitada pola etapa de hidrólise e con porcentaxes entorno ao 30-50%. Para mellorar a dixestión anaerobia das lamas activas pódese aplicar un pre-tratamento (térmico, mecánico, químico ou unha combinación deles), que permita a destrución da matriz da lama (flóculos) para mellorar a hidrólise. Debido a que a lama xerada polos sistemas de biomasa granular aerobia ten unha natureza similar á das lamas activas (procedente dun tratamento biolóxico) o uso da dixestión anaerobia pode tamén ser unha solución interesante para tratala, así como a aplicación dun pre-tratamento antes do proceso anaerobio, que podería contribuír a mitigar o posible efecto do estado de agregación da lama en gránulos. Por iso nos Capítulos 6 e 7 o obxectivo proposto foi estudar a posibilidade de aplicar a dixestión anaerobia para o tratamento da biomasa granular aerobia, primeiro mediante experimentos en descontinuo (Capítulo 6) e logo coa operación dun reactor continuo anaerobio a escala laboratorio (Capítulo 7). En ámbolos dous capítulos estudouse tamén a aplicación dun pre-tratamento térmico para mellorar a dixestión anaerobia da biomasa granular.

Dentro deste contexto a presente tese está enmarcada no campo do tratamento de augas residuais coa aplicación da tecnoloxía baseada en biomasa granular aerobia (Capítulos 3, 4 e 5) e no posterior tratamento da lama granular xerada mediante dixestión anaerobia (Capítulos 6 e 7). Os contidos principais de cada un dos capítulos así como os obxectivos acadados detállanse a continuación:

No Capítulo 1 ofrécese unha visión xeral sobre os procesos de tratamento das augas residuais, centrada no sistema convencional de lamas activas e nos inconvenientes asociados á aplicación desta vella tecnoloxía (gran superficie para a súa implantación, baixa capacidade de tratamento e grandes cantidades de lama xeradas con malas propiedades de sedimentación). Os sistemas de biomasa granular aerobia preséntanse como unha posible alternativa para resolver parte destes problemas. Tamén se realiza unha revisión acerca dos factores que afectan á formación de biomasa granular aerobia así como das súas aplicacións. Finalmente descríbense os aspectos básicos da dixestión anaerobia de lamas con especial atención na microbioloxía do proceso e nos posibles métodos de pre-tratamento.

No Capítulo 2 descríbense os materiais e métodos empregados na tese. Estes engloban a análise das fases líquida, gas e sólida. Algúns parámetros convencionais como o pH, osíxeno disolto, Demanda Química de Osíxeno (DQO), composición do biogás ou concentración de sólidos medíronse de acordo aos métodos estándar. Outras análises realizáronse despois de adaptalas á investigación desta tese, como a caracterización da biomasa granular aerobia mediante o Índice Volumétrico de Lamas (IVL), a determinación da densidade dos gránulos, a aplicación da análise dixital de imaxe para determinar a morfoloxía e o diámetro medio dos gránulos e a medida da concentración de Poly-Hydroxy-Alcanoatos (PHA). Tamén se describe a aplicación da técnica de Hibridación *in situ* con Fluorescencia (FISH) para a identificación das poboacións microbianas involucradas nos procesos biolóxicos, especialmente na dixestión anaerobia.

No Capítulo 3 estudouse o arranque e operación dun reactor secuencial con biomasa granular aerobia para o tratamento dun efluente industrial procedente dunha planta de procesado de produtos do mar. Este efluente industrial estaba caracterizado por unha composición moi variable debido aos diferentes produtos procesados na planta (luras, lagostinos, pescada, etc.). Ademais este efluente procedía dun tratamento físico-químico onde se engadía coagulante e floculante. A operación do reactor granular aerobio estivo dividida en dúas etapas. Na primeira (días 0-295) fomentouse o desenvolvemento dos gránulos aerobios mediante a aplicación de cargas orgánicas entre 2 e 4 kg $DQO_S/m^3 \cdot d$. A partir do día 130 de operación tivo lugar o proceso de granulación e a biomasa granular aerobia desenvolvida presentou boas propiedades de sedimentación, con valores do IVL de 35 mL/g SST e de densidade de 60 g $SSV/L_{gránulo}$ entorno ao día 170 de operación. O diámetro medio dos gránulos ata este momento de operación oscilou entre 2-3 mm, pero logo aumentou ata valores medios de 11.0 mm en poucos días e posteriormente tivo lugar a desintegración dos gránulos, probablemente debido á presenza de maiores concentracións residuais de coagulante e floculante. A eficacia de eliminación da materia orgánica foi do 90% e, aínda que as condicións para a eliminación de nitróxeno non foron optimizadas, acadáronse porcentaxes de eliminación do 65% e 30% de amonio e nitróxeno total, respectivamente. Na segunda etapa (días 296-330) estudouse a estabilidade da biomasa granular aerobia fronte a cargas orgánicas variables (dende 3 ata 13 kg $DQO_S/m^3 \cdot d$) para aproximar a operación do reactor ás condicións reais da industria. Con este test de estabilidade fronte a cargas variables a eficacia de eliminación de materia orgánica non se viu alterada pero as propiedades físicas dos gránulos e a eficacia de eliminación de nitróxeno empeoraron.

No Capítulo 4 operáronse dous reactores secuenciais descontinuos para a obtención de biomasa granular aerobia co obxectivo de coñecer o efecto do coagulante e floculante na formación e características dos gránulos aerobios, posto que os resultados obtidos no Capítulo 3 parecen indicar que estes compostos, que son frecuentemente usados no tratamento primario, poden afectalas. Ámbolos reactores foron alimentados cun medio sintético e un deles ademais foi suplementado cunha cantidade residual de coagulante-floculante (CF), mentres que o outro serviu de control. O principal efecto observado foi unha peor capacidade de retención da biomasa no reactor alimentado con CF comparado co control, o cal implicou unha menor

concentración de sólidos (4.5 vs. 7.9 g SSV/L) e un maior IVL (70 vs. 40 mL/g SST). O diámetro medio dos gránulos aerobios no reactor CF foi maior que no control (5.0 vs. 2.3 mm). Ademais no reactor CF a velocidade máxima de consumo de osíxeno diminuíu respecto ao control pero as eficacias de eliminación de materia orgánica (90%) e nitróxeno (60%) non se viron afectadas.

No Capítulo 5 cambiouse a distribución do ciclo operacional do reactor secuencial descontinuo con biomasa granular aerobia respecto á distribución usada nos Capítulos 3 e 4, para mellorar a capacidade de tratamento e a eliminación de nitróxeno. Nun primeiro experimento empregouse o reactor control do Capítulo 4 con biomasa granular aerobia madura e someteuse a unha diminución progresiva da relación fame/saciedade diminuíndo a lonxitude da fase aerobia. O propósito deste cambio de ciclo foi aumentar a capacidade de tratamento do sistema. Os resultados obtidos mostraron que foi posible reducir a relación fame/saciedade dende 10 ata 5, conseguindo un aumento das cargas orgánica e nitroxenada tratadas no sistema entorno ao 33%. As eficacias de eliminación de materia orgánica (97%) e de nitróxeno (64%) non se viron afectadas mentres que se produciu un lixeiro empeoramento das características dos gránulos. Nun segundo experimento a biomasa granular aerobia madura foi recollida dunha planta piloto (100 L) que trataba xurro de porco e usada para inocular dous reactores secuenciais descontinuos a escala laboratorio. Nestes reactores implantouse unha fase anóxica previa á aerobia para mellorar a eficacia de eliminación de nitróxeno respecto á operación da planta piloto (con só fase aerobia). Os resultados obtidos mostraron que engadindo a alimentación dunha soa vez e cunha fase anóxica de 30 minutos antes da aerobia aumentaba a eficacia de eliminación de nitróxeno no xurro de porco dende o 20% ata o 60%. Non obstante a configuración do ciclo coa alimentación continua e simultánea á fase anóxica de 60 minutos, non só non melloraba a eficacia de eliminación de nitróxeno, senón que mesmo empeoraba a de oxidación de amonio.

No Capítulo 6 estudáronse os efectos do pre-tratamento térmico nas propiedades macroscópicas e bioquímicas do lama granular aerobia, así como a mellora na biodegradabilidade anaerobia despois da aplicación deste pre-tratamento. A caracterización bioquímica das mostras e os resultados obtidos dos ensaios anaerobios en descontinuo tamén foron utilizados para validar un modelo que permita determinar a biodegradabilidade anaerobia da biomasa granular aerobia. Para conseguir estes obxectivos utilizáronse dúas lamas granulares aerobias procedentes de dúas plantas piloto (100 L): unha lama granular dun reactor alimentado con xurro de porco (G1) e outra procedente dun reactor alimentado cun medio sintético para simular unha auga residual urbana (G2). As temperaturas do pre-tratamento utilizadas variaron entre 60-210 ºC para G1 e 170-210 ºC para G2, este pre-tratamento aplicouse durante 20 minutos. Os resultados obtidos para a porcentaxe de biodegradabilidade anaerobia foron do 33% para G1 e do 49% para G2, que son valores similares aos referenciados para lamas activas (30-50%). O pre-tratamento térmico antes da dixestión anaerobia mellorou a biodegradabilidade da lama G1 respecto a mesma lama sen pre-tratamento para todas as temperaturas ensaiadas: 21% a 60 ºC, 42% a 90 ºC, 64% a 115 ºC, 82% a 140 ºC, 88% a 170 ºC, 70% a 190 ºC e 58% a 210 ºC. Para a lama G2 o pre-tratamento

térmico non mellorou a biodegradabilidade a 170º C e só se conseguiu unha mellora do 14% e 18% a 190 e 210 ºC, respectivamente.

No Capítulo 7 estudouse a viabilidade de aplicar a dixestión anaerobia en continuo para o lama granular aerobia. Neste caso a lama granular empregada foi recollida da mesma planta piloto que a lama G1 do Capítulo 6. O dixestor anaerobio empregado tiña un volume útil de 5 L e foi operado en condicións mesofílicas (35 ºC) en tres etapas diferentes. A primeira correspondeu coa dixestión anaerobia da lama granular aerobia sen ningún tipo de tratamento, obténdose valores de biodegradabilidade anaerobia do 44% e de redución de sólidos do 32%, que se atopan dentro do rango dos valores referenciados para lamas activas. A biodegradabilidade anaerobia neste experimento foi maior que a obtida nos ensaios en descontinuo do Capítulo 6 (33%), probablemente debido a cambios na mostra da lama granular aerobia, xa que dende que as mostras foron tomadas da planta piloto entre un experimento e outro transcorreu un ano. A segunda etapa correspondeu coa dixestión anaerobia da lama granular pre-tratada termicamente. Neste caso o pre-tratamento térmico foi levado a cabo a 130 ºC durante 20 minutos. A temperatura de pre-tratamento foi escollida de acordo aos resultados obtidos previamente nos ensaios en descontinuo (Capítulo 6). Este pre-tratamento térmico mellorou o rendemento do reactor nun 32% e 47% en termos de biodegradabilidade e redución de sólidos, respectivamente. Na terceira etapa o dixestor anaerobio foi alimentado cunha mestura de lama granular aerobia pre-tratada termicamente e lama primaria sen tratar, posto que a bibliografía indica que o pre-tratamento da primaria non é necesario. Esta mestura produciu unha mellora do 17% na eliminación de sólidos respecto á lama granular pre-tratada, mentres que a biodegradabilidade diminuíu dende o 58% ata o 53%, o cal foi debido a que a relación $DQO_T$/SV da mestura respecto a só lama granular pre-tratada era maior. Neste capítulo tamén se realizou unha análise das poboacións microbianas dentro do reactor anaerobio mediante a técnica FISH. Os principais feitos observados foron que o dominio *Arquea* tivo unha maior presenza que o dominio *Bacteria*. Ademais a abundancia de ámbolos dominios diminuíu co cambio na alimentación dende lama granular sen tratar, ata lama granular pre-tratada e a súa mestura coa lama primaria.

# Summary

The development of the consumer society is associated to an increase of pollution generation. One of the most important effects is the production of wastewater as a consequence of the human activity (agriculture, industry, business, households, etc.). The discharge of this wastewater without treatment into water bodies can provoke the disappearance of life forms, the loss of the biodiversity, the distortion of water systems, the over-fertilisation and affect the drinking water supplies, which can produce problems in the public health. The treatment of the produced wastewater is therefore necessary to reduce these drawbacks and guarantee its adequate disposal into the environment. However as a consequence of the wastewater treatment another residue is generated, “sludge”, that also needs to be managed.

The biological wastewater treatment is normally accomplished by Conventional Activated Sludge (CAS) systems, which generally require large surface areas for implantation and the presence of biomass separation units due to the poor settling properties of the sludge. Systems based on aerobic granular biomass are an alternative to conventional ones because of their smaller footprint compared to that of the CAS ones. This is due to the fact that the reactor design (large height/diameter ratio) and the properties of the biomass (good settling properties) make unnecessary the construction of secondary settlers. The high concentration of biomass achieved with these systems make it possible to treat large organic and nitrogen loads, which contributes to reduce the necessary volume of the reactor. Furthermore the aggregation of the biomass in granules allows the development of aerobic, anoxic and anaerobic conditions where microbial populations are able to perform different biological processes. This means that in a single unit the simultaneous carbon, nitrogen and phosphorus removal is feasible. Another advantage of the aerobic granular technology respect to the CAS one is the low sludge production of the first one. The management of the sludge produced as a consequence of the wastewater treatment processes accounts for up to 50% of the operational costs of a Wastewater Treatment Plant (WWTP). Furthermore the European regulations applicable to this sludge management are becoming more stringent. Therefore nowadays new technologies are under development in order to, first, reduce the sludge production and, second, improve its further treatment. At this point the aerobic granular biomass based systems are one of the new technologies proposed as substitutes of the CAS ones due to their compact configuration.

The first works that reported the formation of aerobic granular biomass date from the 90’s. So far research has been focused in the improvement of the different aspects related to the full scale application of this technology. It is known that a number of factors affect the granulation process, being one of them the type of substrate. Up to date, the results from several research

works seem to indicate that the formation of aerobic granules is possible treating different substrates but evidences show that the microbial diversity and the structure of mature granules are closely related to them. The development of aerobic granular biomass has been studied treating different synthetic mediums and industrial wastewaters, which indicates that it is possible to grow aerobic granules with complex substrates. This is of interest for the industries with implantation surface limitations for the WWTP installation. This limitation is more evident in the case of those industries located near the coast, which are normally dedicated to the processing of seafood (canning, aquaculture, etc.). In this sense in Chapter 3 the development of aerobic granular biomass with the effluent from this type of industry has been studied, paying special attention to the characteristics of the formed aerobic granules and to the achieved efficiencies of organic matter and nitrogen removal. The stability of the aerobic granular biomass to sudden changes of the applied load was also studied, since the effluents from the seafood industry are characterized by a high variability in composition.

The influence of different specific compounds on the process, not only the determination of the usefulness of the technology applied to a specific effluent, also is important. The effects of the presence of compounds like phenol, pyridine, heavy metals and dyes have been studied on aerobic granular systems. However the effects of the presence of coagulant-flocculant reagents, commonly used in the WWTPs as pre-treatment for solids separation before the biological processes, have not been studied so far. As the industrial effluent used in Chapter 3 was produced in a pre-treatment unit where coagulant and flocculant reagents were added, the obtained results seem to indicate that the residual concentration of these compounds could affect the properties of the aerobic granular biomass. For this reason in Chapter 4 the effects of these compounds on aerobic granular systems were tested using as effluent a synthetic medium to avoid the interference of another complex substances present in the seafood industrial effluent.

The aerobic granular systems are operated in Sequencing Batch Reactors (SBRs) with a short initial feeding phase followed by an aerobic reaction phase that allows submitting the biomass to feast-famine regimes, where the feast period is short and the famine one long. These conditions are suitable to select the appropriate microorganisms to obtain compact and dense granules. However an excessively long famine period may not be necessary and lead to an extra energy consumption and low reactor capacity. Furthermore the nitrogen removal efficiency is limited by the denitrification process due to the high dissolved oxygen (DO) concentration obtained along the famine phase. Therefore in Chapter 5 other cycle configurations were tested in order to establish the length and the distribution of the operational cycle which allow maximizing the treatment capacity and the nitrogen removal efficiency, respectively.

Although the production of sludge with the aerobic granular systems, where organic matter and nitrogen are simultaneously removed, is lower than with the CAS process, its treatment is still necessary. However, nowadays, no much research about its possible post treatment is available. This lack of information is due to the fact that the aerobic granular systems are at the moment under study at pilot scale. In order to scale up the process, and previously to the

industrial application, the study of the potential treatability of this type of sludge is of great interest. The anaerobic digestion is widely used for the treatment of the sewage sludge generated in WWTPs because it allows stabilizing its organic content reducing the solids and producing biogas that can be recovery as energy. However the anaerobic degradation of the excess sludge produced in the CAS process (Waste Activated Sludge, WAS) is normally limited by the hydrolysis step rate and the maximum achievable percentage values are between 30-50%. To enhance the anaerobic digestion of the WAS a pre-treatment can be applied (thermal, mechanical, chemical or a combination of them), which leads to a destruction of the sludge microbial matrix (flocs) to improve the hydrolysis rate. Since the sludge produced in the aerobic granular systems has a similar nature to the WAS (coming from a biological treatment) the use of the anaerobic digestion can be also an interesting solution to treat it, as well as the application of a pre-treatment before the anaerobic step, which could contribute to mitigate the possible effect of the state of aggregation of the biomass in granules. Therefore in Chapters 6 and 7 the objective was to study the feasibility of the anaerobic digestion to treat the Aerobic Granular Sludge (AGS), first performing batch experiments (Chapter 6) and then with the operation of a continuous anaerobic digester at laboratory scale (Chapter 7). The application of a thermal pre-treatment to improve the anaerobic digestion of AGS also was studied in both chapters.

In this context the present thesis is framed in the field of wastewater treatment by the application of the aerobic granular technology (Chapters 3, 4 and 5) and the posterior treatment of the granular sludge generated by anaerobic digestion (Chapters 6 and 7). The main contents of each of the chapters comprising this thesis and the achieved objectives are detailed in the following sections:

In Chapter 1 an overview about the wastewater treatment processes is provided, focused on the CAS and the bottlenecks associated to the application of this old technology (high surface requirements, low load capacity and large amounts of sludge production with poor settling properties). The aerobic granular systems are presented as a possible alternative to solve part of these drawbacks. A review of the factors affecting the formation of aerobic granular biomass and the applications of this technology are presented. Finally the basic aspects of the sludge anaerobic digestion are described with special attention paid to the microbiology of the process and the different available pre-treatment methods.

In Chapter 2 the materials and methods used in the thesis are described. They comprised the analysis of the liquid, gas and solid phases. Some conventional parameters like the pH, dissolved oxygen (DO), chemical oxygen demand (COD), biogas composition or solids concentration were measured following the instructions of the Standard Methods. Another analysis were performed after being adapted to the research of this thesis, like the characterization of aerobic granular biomass by Sludge Volume Index (SVI), determination of the granules density, the application of the digital image analysis to determine the morphology and the average granule diameter and the measurement of the Poly-Hydroxy-Alkanoates (PHA) concentration. The Fluorescent *in situ* Hybridization (FISH) technique, applied to the

identification of the microbial populations involved in the biological processes, especially in the anaerobic digestion, is also described.

In Chapter 3 the start-up and operation of an aerobic granular SBR treating the industrial effluent from a seafood processing plant were studied. This industrial effluent was characterized by a high variable composition due to the different products processed in the plant (squid, prawn, hake, etc.). Furthermore this effluent came from a previous physical-chemical pre-treatment where coagulant and flocculant reagents were added. The operation of the aerobic granular SBR was divided in two stages. In the first one (days 0-295) the development of the aerobic granules was promoted using applied Organic Loading Rates (OLRs) between 2 and 4 kg $COD_S/m^3 \cdot d$. The granulation process took place after 130 days of operation and the developed aerobic granular biomass exhibited good settling properties, with values of the SVI of 35 mL/g TSS and the density of 60 g $VSS/L_{granule}$ around day 170 of operation. The granules presented an average diameter between 2-3 mm, however the presence of high residual concentrations of coagulant-flocculant reagents might be the responsible for the increase in the average granule diameter to 11.0 mm occurred in few days, which led to the disintegration of the aerobic granules. The efficiency of organic matter removal was of 90% and, although the conditions for nitrogen removal were not optimized, percentages of 65% and 30% for ammonia and total nitrogen removal, respectively, were achieved. In the second stage (days 296-330) the stability of the aerobic granular biomass when variable OLRs were applied (from 3 to 13 kg $COD_S/m^3 \cdot d$) was tested in order to approximate the operation of the SBR to the real conditions in the industry. The stability tests with variable OLRs showed that the organic matter removal efficiency was not affected but the granules physical properties and the nitrogen removal efficiency experienced a detrimental effect.

In Chapter 4 two aerobic granular SBRs were operated in order to know the effect of the coagulant-flocculant reagents on the formation and characteristics of aerobic granules, since the results obtained in Chapter 3 seem to indicate that these compounds, frequently used in the primary treatment, can affect them. Both SBRs were fed with a synthetic medium and one of them was supplemented with a residual amount of coagulant-flocculant reagents (CF), while the other served as control. The principal observed effect was a worse biomass retention capacity of the reactor fed with CF compared to the control one, which implied lower solids concentration (4.5 vs. 7.9 g VSS/L) and higher SVI (70 vs. 40 mL/g TSS). The granules obtained in the CF reactor presented also a higher average diameter (5.0 vs. 2.3 mm). Another observation was that in the CF reactor the maximum oxygen consumption rate decreased respect to the control but the removal efficiencies of organic matter (90%) and nitrogen (60%) were not affected.

In Chapter 5 the distribution of the operational cycle of the aerobic granular SBR was changed respect to the distribution used in Chapters 3 and 4 in order to improve the treatment capacity and the nitrogen removal. In a first experiment the mature aerobic granular biomass of the control reactor of Chapter 4 was submitted to a progressive decrease in the famine/feast ratio by decreasing the length of the aerobic phase. The aim of this cycle change was to increase the treatment capacity of the system. The results obtained showed that to reduce the famine/feast ratio from 10 to 5 was possible increasing the treated OLR and Nitrogen Loading

Rate (NLR) in the system around 33%. The removal efficiencies of organic matter (97%) and nitrogen (64%) were not affected while a slight detriment of the granules characteristics was produced. In a second experiment mature aerobic granular biomass was taken from a pilot plant (100 L) treating pig manure and used to inoculate two SBRs at laboratory scale. In these SBRs an initial anoxic phase, previous to the aerobic one, was implemented in order to improve the nitrogen removal efficiency respect to the operation of the pilot plant (with only an aerobic phase). The results obtained showed that an anoxic phase of 30 min previous to the aerobic one with a pulse-fed mode increased the removal efficiency of the nitrogen in the pig manure from 20 to 60%. However the cycle configuration comprising a continuous feeding simultaneous to an anoxic phase of 60 min not only did not enhance the nitrogen removal efficiency but also worsened the ammonia oxidation one.

In Chapter 6 the effects of the thermal pre-treatment on the macroscopic and biochemical characteristics of the AGS were studied, as well as the anaerobic BioDegradability (BD) enhancement after this pre-treatment application. The biochemical characterization of the samples and the results obtained from the batch anaerobic tests were also used to validate a model that allows estimating the anaerobic biodegradability of the aerobic granular biomass. To achieve these objectives two AGS samples from aerobic granular pilot plants (100 L) were studied: one from a reactor fed with pig manure (AGS1) and another from a reactor fed with a synthetic medium to simulate an urban wastewater (AGS2). The pre-treatment temperatures were tested in a range between 60-210 ºC for AGS1 and 170-210 ºC for AGS2 and applied during 20 minutes. The results obtained with the untreated AGS samples showed that their anaerobic BD, of 33% for AGS1 and 49% for AGS2, was similar to that reported for a WAS (30-50%). The thermal pre-treatment before the anaerobic digestion enhanced the BD of AGS1 respect to the untreated sludge for all the temperatures assayed: 21% at 60 ºC; 42% at 90 ºC; 64% at 115 ºC; 82% at 140 ºC; 88% at 170 ºC; 70% at 190 ºC and 58% at 210 ºC. For AGS2 the thermal pre-treatment did not enhance the anaerobic BD at 170 ºC and improvement of only 14% and 18% were achieved at 190 and 210 ºC, respectively.

In Chapter 7 the feasibility of the application of continuous anaerobic digestion of AGS was studied. In this case the AGS sample was collected from the same aerobic granular pilot plant than AGS1 in Chapter 6. The anaerobic digester had a useful volume of 5 L and was operated in the mesophilic range (35 ºC) in three different operational stages. The first one corresponded with the anaerobic digestion of raw AGS, where the obtained values of BD (44%) and solids reduction (32%) were in the range than those reported for WAS. The anaerobic BD in this case was higher than that obtained in the batch tests (33%) in Chapter 6, probably due to changes in the AGS sample, since samples used in both experiments were collected from the pilot plant with one year of difference. The second stage corresponded with the anaerobic digestion of thermal pre-treated AGS. In this case the thermal pre-treatment was carried out at 130 ºC along 20 minutes. The temperature of pre-treatment was chosen according to the results obtained previously in the batch tests (Chapter 6). This thermal pre-treatment of the AGS enhanced the reactor performance 32% and 47% in terms of BD and solids reduction, respectively. In the third stage the anaerobic digester was fed with a mixture of thermal pre-treated AGS and raw Primary

Sludge (PS), since the bibliography indicates that the pre-treatment of the last one is not necessary. This mixture produced an enhancement of 17% for the solids removal respect to only pre-treated AGS, while the BD decreased from 58% to 53%, which was due to the higher $COD_T$/VS ratio of the mixture respect to that corresponding to the experiment with only pre-treated AGS. In this chapter microbial analysis with the FISH technique were done to know how the type of feeding influences the development of different microbial populations inside the anaerobic reactor. The main features observed were that the *Archaea* domain had a higher presence in comparison with the *Bacteria* domain. Furthermore the abundance of both domains decreased with the change on the feeding from raw AGS, to pre-treated AGS and its mixture with PS.

# Chapter 1:
# Introduction


## Summary

This chapter aims to present the scope of this thesis. In a first point a brief overview about the wastewater, production and treatment, is provided to evaluate the convenience of the substitution of conventional treatment systems based on activated sludge by another with a smaller footprint, more efficient in terms of treatment capacity and nitrogen removal and with a lower sludge production.

The aerobic granular technology is presented as a good alternative of improvement and a short historical evolution, conditions of operation, advantages, applications and scale-up are provided. Although this is a recent technology and some aspects like the stability of the formed granules, the optimization of the operation and the post-treatment of the aerobic granular sludge (AGS) generated are not still completely known.

In a last part of the chapter the basic aspects of the anaerobic digestion and the possible pre-treatments available to improve it are described in order to know its applicability to the AGS digestion. The anaerobic digestion is an old and well-known process to treat the sewage sludge, so it is considered as a suitable option to treat the AGS.

## 1.1. CONVENTIONAL ACTIVATED SLUDGE PROCESS

### 1.1.1. Wastewater: production and treatment

In the last century, the global water use has increased by a factor of six with differences among the developed and developing areas. This high increase in water use is due to the global population growth and economic development. Due to this raise of wastewater production its treatment and disposal is an increasing drawback.

The wastewater treatment is normally performed applying sequential treatments which correspond to three different types: primary, secondary, and tertiary (or advanced) treatments (Metcalf and Eddy, 2003). The primary treatments remove material that will either float or readily settle out by gravity and include physical processes like screening, grit removal and sedimentation. With the primary treatments approximately 50 to 70% of Total Suspended Solids (TSS), 25 to 50% of organic matter and 65% of the oil and grease are removed. The secondary treatments remove more than 85% of both TSS and organic matter in wastewater by using biological treatment processes. From this group the suspended growth systems like Conventional Activated Sludge (CAS) process are the most common ones. Although the primary and secondary treatments remove the majority of TSS and organic matter in most of the cases this level of treatment has proved to be insufficient to protect the receiving waters or to provide reusable water for industrial and/or domestic recycle. Thus, additional treatment stages, called advanced or tertiary treatments, have been included in the Waste Water Treatment Plants (WWTPs) to provide further organic and solids removals or nutrients and/or toxic materials removal. Tertiary treatment technologies can be considered as extensions of conventional secondary biological treatments or involve physical-chemical separation techniques (carbon adsorption, membranes, ion exchange, de-chlorination, reverse osmosis, etc.)

In the European Union (EU) the wastewater treatment is regulated by the Directive 91/271/EEC for Urban Waste Water Treatment (CEC, 1991), that has as objective to protect the environment from the adverse effects of urban wastewater disposal and water discharges from certain industrial sectors. The degree of treatment depends on the zone of disposal. In case of agglomerations with more than 2,000 population equivalents (p.e.) the secondary treatment must be applied for all discharges while for agglomerations with more than 10,000 p.e. in designated sensitive areas and their catchments advanced treatments are required. According to estimations of the EU-27 (CEC, 2009) there are more than 23,000 agglomerations larger than 2,000 p.e., which produce a total wastewater pollution load of about 600 million p.e. and a surface of about 68% of the EU-27 territory is considered as sensitive area. As a result of the progressive implementation of this directive the amount of treated wastewater has increased in the EU in the last years (Figure 1.1).

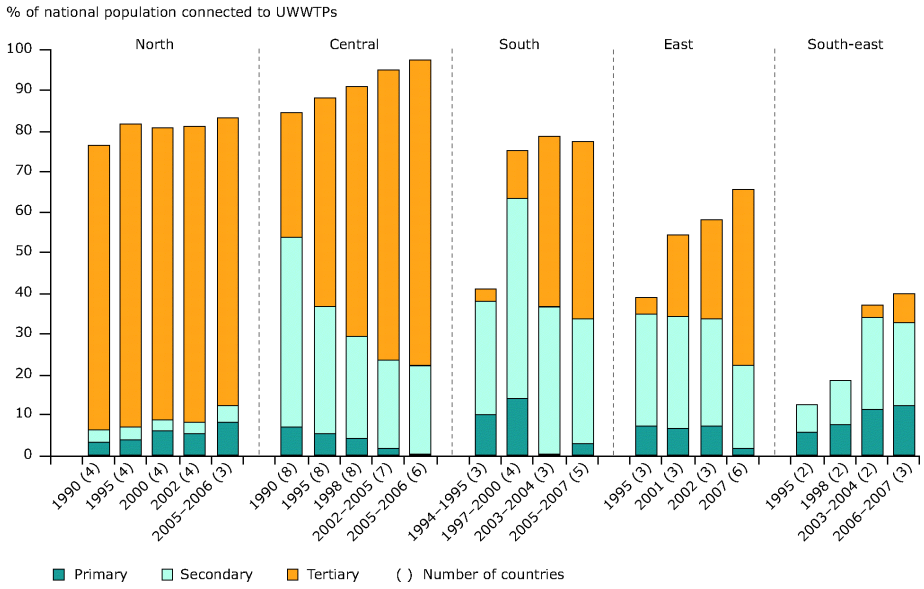


**Figure 1.1.** Population percentage per European region connected to an urban WWTP over the period 1990 to 2007 and type of applied treatment (Source: European Environmental Agency and Eurostat).

### 1.1.2. Limitations of the Conventional Activated Sludge (CAS) process

As it was previously mentioned the most widely used secondary treatment in the WWTPs is the CAS process (Figure 1.2). The system consists of an aerated tank that contains a suspension of microorganisms in wastewater. The organic matter oxidation and, under certain conditions, the nitrogen removal, take place in the biological reactor by means of the activity of the microorganisms, which grow using the substrate present in the wastewater. Completely mixing inside the aerated tank is achieved by means of aeration devices which also supply the needed oxygen for the microbial suspension. Once the suspension leaves the aeration tank enters the secondary settler where the microorganisms are separated from the liquid (effluent) by sedimentation. A portion of the settled biological sludge is then recycled to the aeration tank to maintain a high Mixed Liquor Suspended Solids (MLSS) concentration and the excess is purged from the system and sent to the sludge treatment line. However, despite the worldwide accepted use of this simple technology for hundreds of years, it presents some limitations related to its maximum removal capacity (Campos *et al.*, 1999), nitrogen removal efficiency (Kim *et al.*, 2011) and sludge production (Ramdani *et al.*, 2010).

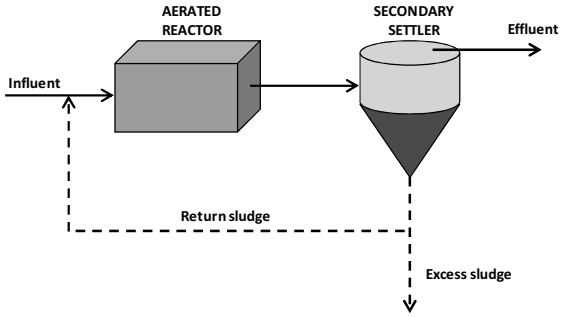


**Figure 1.2.** Schematic layout of the main units of the CAS process.

***System capacity***

Normally the CAS process capacity is limited by the maximal biomass concentration that can be accumulated. Due to the common poor settling properties of the activated sludge, with Sludge Volume Index (SVI) values normally above 100 mL/g TSS and settling velocities around 1 m/h, the biomass concentration achieved inside the aerated reactor is low (1.5-3.5 g VSS/L) (von Sperling, 2007). This fact limits the treatment capacity to Organic Loading Rates (OLRs) between 1-2 kg COD/$m^3$·d, which implies the use of large reactors. Maintenance of higher biomass concentrations in the system requires the installation of bigger secondary settlers which supposes a very high area requirements and an important increase of investment capital costs.

Additionally, problems of bulking can appear due to the proliferation of filamentous microorganisms that worsen the settling properties of the flocs and promote the formation of foams in the surface of the secondary settler, producing high concentrations of suspended solids in the effluent (Seka *et al.*, 2001).

***Nitrogen removal***

The nitrogen removal in the CAS process is performed by means of the nitrification and denitrification processes. Nitrification converts the ammonia to nitrate and denitrification reduces nitrate to nitrogen gas.

Conventional processes used for nitrogen removal, nitrification and denitrification, take place in different environmental conditions. The denitrification process requires the absence of oxygen and the presence of organic matter and nitrate, while the nitrification occurs in the presence of oxygen in aerated systems. The most common configuration to achieve the nitrogen removal is the pre-denitrification one, which consists in a reactor tank comprising an anoxic zone previous to the aerated zone. The nitrification occurs in the aerobic zone, leading to the formation of nitrate. Nitrate is directed to the anoxic zone by means of an internal recirculation with high recycle ratios (from 100 to 400% of the influent flow). In the anoxic zone nitrate is reduced into nitrogen gas using as carbon source the wastewater that enters the system. The disadvantage of this configuration is that, to reach high denitrification efficiencies, very high

internal recirculation ratios are needed, which is not always economically advisable (von Sperling, 2007).

To achieve an efficient nitrification in the CAS process, relatively long Solids Retention Time (SRT) is necessary due to the fact that the growth rate of the nitrifying organisms is much slower than that of the heterotrophic organisms responsible of the organic matter oxidation (von Sperling, 2007). Thus the SRT should be such that it enables the development of the nitrifying bacteria before they are washed out from the system. Taking into account that the maximum specific growth rate of the nitrifying bacteria is between 0.3-0.7 $d^{-1}$, Arceivala (1981) proposes that for wastewater without any specific inhibiting factor the minimum SRT value for nitrification with temperatures between 5 and 20 ºC is 12 and 3.5 days, respectively. Although to include a safety factor in the order of 1.5 to 2.5 is recommended to overcome possible peaks of influent ammonia load and other unexpected environmental variations (von Sperling, 2007). This long SRT requirement implies a larger reactor volume since the biomass concentration of CAS process is limited. Furthermore the treated Nitrogen Loading Rate (NLR) is low due to the low biomass concentration maintained in the unit able to perform the nitrogen removal processes (Campos *et al.*, 1999).

***Sludge production***

As a consequence of the wastewater treatment an unwanted by-product is generated: sludge. The disposal of this sludge is both difficult and expensive, accounting between 50 and 60% of the operational costs in a WWTP (Vesilind and Spinosa, 2001). The net sludge production in an activated sludge process depends on the amount of solids contained in the wastewater that enter the system (pre- settled or not) and of the SRT in the CAS unit. High concentration of solids in the influent are associated to high sludge production while long SRT values involve a low quantity of generated Waste Activated Sludge (WAS). Taking into account that the typical values for SRT in a CAS process are between 4 and 10 days and that the primary treatment is often performed, the net sludge production in an activated sludge process is around 0.25-0.3 g VSS/g $COD_{removed}$ (Ginestet, 2007).

The excess sludge produced in the CAS process is significantly different from Primary Sludge (PS). The WAS is typically the most difficult sludge to dewater because it contains so much water initially (99%) (Ramdani *et al.*, 2010) and because the type of water in the sludge is attached by chemical and physical means to the surface area provided by the tiny microorganisms. WAS does not contain the concentration of pathogens found in PS, but its inability to dewater and its high concentration of volatile solids make it difficult to treat in the WWTP (Vesilind and Spinosa, 2001).

Therefore, to solve the problem associated to the high production of sludge in the CAS process, to develop alternative technologies to reduce the sludge production in origin and to improve the post-treatment processes is necessary (Figure 1.3), which allow reducing costs of dewatering, transport or drying and to enhance sludge biodegradation.

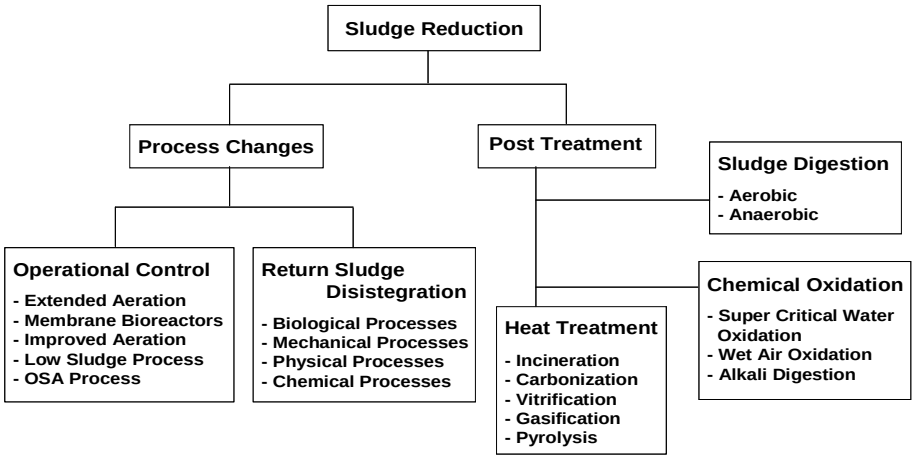


**Figure 1.3.** Outline of sludge reduction technologies (adapted from Mahmood and Elliott, 2006).

In this sense different technologies are proposed to reduce the sludge production in origin and substitute the CAS (Campos *et al.*, 2009; Foladori *et al.*, 2010). An example is the use of membrane bioreactors that work with long SRT and low Food to Microorganisms (F/M) ratios to promote the biomass lysis, allowing the proliferation of higher organisms which graze bacteria causing less sludge production. The oxic-settling-anaerobic process, that is a modification of the CAS by inserting an anaerobic concentrated sludge tank in the recycling bypass, also reduces the excess sludge production by 23% to 58% (Saby *et al.*, 2003; Ye and Li, 2005). Another option is the extended aeration process that consists of a CAS process which operates at a low F/M ratio to maintain biomass under the endogenous respiration phase reducing to 50% the excess sludge produced. Other alternatives could be the use of the low sludge production process with a reduction in the overall biomass production between 12–43% (Ratsak *et al.*, 1994); the application of sludge disintegration techniques to enhance the hydrolysis rate of particulate matter, which is the limiting step of the solids degradation; and the use of Aerobic Granular Sludge (AGS), which can reduces the amount of sludge generated and its volume as well as improve the subsequent anaerobic sludge digestion (Campos *et al.*, 2009).

Despite all these alternatives to reduce the sludge production there is still the need of its further treatment before disposal in order to transform the amount of highly degradable organic matter into a stable residue and to reduce the number of disease-causing microorganisms present. The type of applied treatment can vary and depends on the final chosen disposal option of the sludge (Houdková *et al.*, 2009). The aerobic digestion prevails mostly in small scale WWTPs and the anaerobic digestion is the most widely used process in large WWTPs with capacity above 20,000-30,000 p.e., where the energy recovered by exploiting the produced methane gas becomes economically advantageous (Foladori *et al.*, 2010). Drying is preferred when the sludge is to be incinerated; however it is only profitable if fossil fuels are not required. Other options to be considered are solar drying or utilization of waste heat coming from different processes.

## 1.2. ALTERNATIVES TO THE CAS PROCESS

In view of the CAS limitations exposed previously some technologies are presented as good alternative: the membrane bioreactors, the biofilm systems and the aerobic granular systems.

### 1.2.1. Membrane Biological Reactors (MBRs)

In the Membrane Biological Reactors (MBRs) the separation of sludge and effluent takes place by Micro- or Ultra-Filtration (MF/UF) in a highly efficient membrane module rather than in a conventional gravity settler. The membrane can be located outside of the aerated reactor or immersed (Figure 1.4), this last option is more frequent. The main advantages of MBR compared to CAS process are: (i) high efficiency in TSS removal, (ii) application of high sludge concentrations in the reactor, (iii) reduced sludge production and (iv) small footprint (Foladori *et al*., 2010).

Efficiencies close to 100% of solids separation can be obtained when using membranes which also serve as advanced treatment systems for coliform bacteria removal. The use of membranes produces a MF/UF quality effluent with a very low TSS concentration suitable for reuse applications. Indicative output quality of MF/UF systems include SS < 1 mg/L, turbidity <0.2 NTU and up to 4 log removal of virus (depending on the membrane nominal pore size). In addition, it provides a barrier to certain chlorine resistant pathogens such as *Cryptosporidium* and *Giardia* (Leslie and Chapman, 2003).

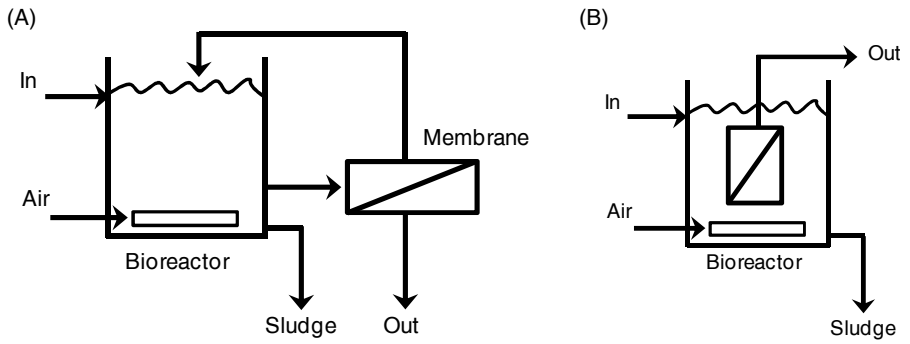


**Figure 1.4.** Configurations of a MBR: (A) sidestream and (B) immersed (Judd, 2011).

As the separation of the treated water from the biological sludge takes place in the membrane and the concentration of the solids in the effluent is very low, secondary settlers and tertiary filtration processes are eliminated, thereby reducing plant footprint. Furthermore the solids concentration achieved inside the aerated reactor is higher (7-20 g TSS/L) than in a CAS which allows the decrease of the reactor volume.

Due to the good separation of solids in the membrane the hydraulic and solids retention times can be completely decoupled, independently of the settling properties of biomass. Then

the maintaining of high solids contents inside the bioreactor is possible and long SRT and low F/M ratio are achieved without negative effects on the effluent quality (Masse *et al.*, 2006; van Nieuwenhuijzen *et al.*, 2008). The long SRT values achieved together with a low F/M ratio promote the biomass lysis (Lobos *et al.*, 2005) and also allow the proliferation of higher organisms which graze bacteria (Ghyoot and Verstraete, 2000), causing less sludge net production. Zero sludge production have been reported at high sludge concentration (around 20 g TSS/L) and F/M ratios about 0.07 kg COD/kg TSS (Rosenberger *et al.*, 2000).

Despite of the advantages of the MBR systems, the maturity of the technology and its wide application around the world, some drawbacks are still unsolved like fouling, high energy consumption compared to CAS process and high investment costs (Lesjean *et al.*, 2011). Membrane fouling is caused by the deposition of biosolids onto the membrane surface, which leads to a decrease in flux and an increase in aeration demand, which in turn leads to more frequent membrane cleaning and replacement actions. These operations increase the operational costs. Furthermore the operation at long SRT values with high biomass concentrations inside the system contributes to the fouling which provokes the decrease in both oxygen transfer efficiency and membrane permeability (Le-Clech *et al.*, 2003; Yigit *et al.*, 2008). In practice, it is not feasible to operate a MBR with complete sludge retention. The optimal operational conditions should be defined taken into account sludge treatment, aeration and membrane cleaning/replacement costs.

### 1.2.2. Biofilm systems

The restrictions in the available surface for the installations of the WWTPs make the development of compact processes, capable to treat high loads in small space, necessary. An interesting option is the use of biofilms systems based on the development of the biomass attached to a support material, instead of growing in suspension in the aerated tank. Biofilm systems are more compact and present lower surface impact than the CAS systems when installed in urban areas (Rogalla *et al.*, 1992) and, above all, they are highly resistant to variations in temperature and to toxicity shock loads (Arvin and Harremoes, 1990). The biofilm systems have been used to upgrade overloaded treatment plants, because the applied organic load can be up to three times higher compared to that achieved in the CAS process (Lessel, 1993). Also the fact that the biomass grows adhered to a carrier material allows that its retention capacity do not depend on the settling characteristics of the biomass (Tijhuis *et al.*, 1994).

The biofilm systems can be classified, according to the microbial aggregation, in static biofilms (e.g. trickling filters, rotating biological contactors and fixed bed reactors) or suspended biofilms (e.g. biofilm fluidized bed reactor and biofilm airlift suspension reactors). In the suspended biofilms the biomass grows on a carrier material that it is in permanent movement (hydraulically or mechanically driven) and generally possesses a large specific surface area for the attachment of the biomass. This support material can be in the form of grains of small diameter (0.2 to 2.0 mm) or a material with high porosity (e.g. sponges). In the 90's of the past century the use of these systems was promoted and airlift or fluidised bed reactors were widely

studied (Heijnen *et al.*, 1990; Tijhuis *et al.*, 1996). The main advantages of the suspended biofilms in comparison with the CAS process are (Nicolella *et al.*, 2000): (i) higher terminal settling velocity of solids (50 m/h) in comparison with flocculated sludge (5 m/h), leading to possible the elimination of secondary settlers; (ii) the biomass concentration achieved inside the reactor is high (> 20 kg TSS/$m^3$) resulting in a high treatment capacity; (iii) compact reactor with small area requirements is used because the process can be operated at high biomass concentration without the need of settlers for biomass retention and recirculation; (iv) high biomass age (several weeks) and minimization of excess sludge production.

However one disadvantage of the biofilm systems is the high operational and maintenance costs compared with CAS system related to the necessity of an appropriate flow distribution and aeration, as well as the use of a carrier material.

### 1.2.3. Aerobic granular systems

Aerobic granular biomass consists of compact and dense microbial aggregates with a spherical outer shape, which represent a particular or special case of biofilm development where a carrier material is not needed (Liu and Tay, 2004). The main advantages of this system compared to the CAS one are: (i) lower surface requirements, (ii) higher treatment capacity and (iii) lower sludge production.

The aggregation in dense and compact granules with high settling velocities allows the accumulation of high amounts of active biomass that can settle in the same reactor without the need of secondary clarifiers (Beun *et al.*, 1999). Furthermore the reactor is designed with a large High/Diameter (H/D) ratio, which contributes to the reduction of the footprint compared with the CAS process. The biomass aggregation into granules can also reduce the surface requirements because in a single unit the simultaneous removal of different compounds (organic carbon, nitrogen and phosphorus) is possible (Lin *et al.*, 2003; Cassidy and Belia, 2005; de Kreuk *et al.*, 2005a).

The high concentrations of biomass achieved with the aerobic granular system in comparison with the CAS process allow treating higher substrate loading rates. Previous research works show that aerobic granules can treat a very wide range of OLRs between 1.5-19.5 kg COD/$m^3$·d (Moy *et al.*, 2002; Liu *et al.*, 2003a; Adav *et al.*, 2010) while the typical values for a CAS process range between 0.5-1.0 kg COD/$m^3$·d.

As it was previously mentioned the net yield sludge production for an aerobic granular system is expected to be lower than for a CAS process. Tay *et al.* (2001b) observed that the sludge production of granular systems was 30% lower than in activated sludge systems. A possible explanation is that microorganisms which grow forming granules have a higher percentage of exopolymers in their composition to maintain their structure. The production of these compounds implies a change of their metabolism and more energy is used in this process compared to flocculant microorganisms. Furthermore the volume of sludge produced is lower due to the denser and compacter structure of the granules compared to flocs. Then the

subsequent treatment of the sludge is easier since most of the processes applied are focused on reducing its volume by decreasing its water content. On the other hand, during granulation cells increase their hydrophobicity from 50 to 80% which can favour dewatering processes (Tay *et al.*, 2001b). Also an improvement of the anaerobic sludge digestion is expected due to the high content of Poly-Hydroxy-Butyrate (PHB) in the bacterial cells which is known to be a biodegradable organic matter storage compound (Fang *et al.*, 2009).

Despite all these advantages the aerobic granular biomass is a recent technology and more research work is necessary to go into aspects like the physical stability of the granules, the optimization of the removal processes (e.g. nitrogen) and the subsequent treatment of the generated AGS.

## 1.3. AEROBIC GRANULATION

The first works that reported the formation of granular biomass were carried out with anaerobic processes. The Upflow Anaerobic Sludge Blanket (UASB) reactors acquired popularity because they allowed the obtaining of granular biomass with high settling velocity and the accumulation of high concentrations of solids inside the reactor systems (Lettinga *et al.*, 1980; Fang *et al.*, 1995). To achieve the same advantages but with aerobic processes the first works of sludge granulation in aerobic conditions has been 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) reactor 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 the repercussion of this work was scarce. Later Morgenroth *et al.* (1997) showed the basis to obtain aerobic granular biomass in a Sequencing Batch Reactor (SBR) with very short sedimentation and draw phases, which supposed an enhancement in the aerobic processes operation similar to that previously obtained with the anaerobic processes and opened the door to other works with aerobic granular biomass (Beun *et al.*, 1999; Dangcong *et al.*, 1999; Beun *et al.*, 2000; Tay and Liu, 2001; Tay *et al.*, 2002b).

From the results obtained in these initial works and due to the high diversity in the characteristics of the obtained aerobic granular biomass, the necessity to establish a definition to discern between an aerobic granule and a simple floc with relatively good settling properties emerged. This definition of “aerobic granule” came out from the discussions which took place at the “*1st IWA-Workshop Aerobic Granular Sludge*” in Munich (2004) and 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.*, 2005b).

### 1.3.1. Conditions to obtain aerobic granular biomass

Since the 90's, when the first research about aerobic granulation appeared, to nowadays, a multitude of works were performed with different operational conditions in the field of aerobic granulation. Obviously to obtain aerobic granular biomass a number of conditions have to be fulfilled. Existing literature on AGS typically focuses on a few parameters that are identified to influence granule formation, although the major part of the works evaluate these factors separately.

***Reactor configuration***

So far, most research on aerobic granulation has been conducted in Sequencing Batch Reactors (SBRs). Compared to continuous operated reactor systems, the main feature of SBR systems is its cycle operation. Each operation cycle consisted of different phases: filling, aeration, settling, discharging and idle one, although this last phase is the transition between cycles and it is optional. The SBR system has some advantages in comparison with the continuously operated reactors because the settling phase substitutes the performance of the settler (less surface requirement) and also save the pump cost of the biomass recirculation. Furthermore the discontinuous operation prevents fluctuated loads. The operation with SBR also allows choosing short settling times to exert a selection pressure on the sludge particles, i.e. only particles that can settle down within the given settling time are retained in the reactor and otherwise they would be washed out of the system.

The aerobic granulation in continuous systems was tested by Morales *et al.* (2010). These authors achieved the aggregation of the biomass and although the physical properties were not suitable for aerobic granules, with high SVI (133 mL/g TSS) and low density (11g VSS/$L_{granule}$), supposed an improvement respect to the settleability of activated sludge, because parameters like low settling times and Hydraulic Retention Times (HRTs) were imposed to promote the wash-out of suspended biomass. Although at the moment no successful aerobic granulation has been observed in continuous systems.

Associated with the settling time a reactor with a large H/D ratio is advantageous, because the settling time could be regulated to values low enough to retain only the biomass with high settling velocity and led also to a reactor with a small footprint (Beun *et al.*, 2002b). Furthermore a large H/D ratio can ensure a longer circular flowing trajectory, which in turn creates an optimal interactive pattern between flow and microbial aggregates for granulation (Yu, 2006).

***Settling time***

Strategies to promote the formation of aerobic granules include the use of short settling times. This hydraulic selection pressure on the microbial community allows the retention of granular biomass inside the reactor while the flocculent biomass is washed-out. Qin *et al.* (2004a; 2004b) studied the effect of this parameter on aerobic granulation in SBR systems and found that aerobic granules were successfully cultivated and became dominant only in SBRs operated at settling times shorter than 5 minutes which corresponded with a minimal settling

velocity of 8 m/h, while a mixture of aerobic granules and suspended sludge developed in SBRs run at longer settling times.

In aerobic granulation research, a short settling time has been commonly used to enhance aerobic granulation in SBR (Jiang *et al.*, 2002; Lin *et al.*, 2003; Liu *et al.*, 2003a; Yang *et al.*, 2003; McSwain *et al.*, 2004; Linlin *et al.*, 2005; Liu *et al.*, 2005b). In fact, when long settling times are applied, poorly settling sludge flocs cannot be effectively withdrawn; and they may overtake granule-forming microorganisms. As a result, aerobic granulation can fail in SBR systems run at too long settling times. This seems to indicate that aerobic granules can be formed only at short settling times below a critical settling velocity level, being a decisive factor in the formation of aerobic granules in SBR.

***Feast-famine regime***

From previous research works it is known that to promote the formation of AGS, in systems where oxidation of organic matter takes place, short feeding periods must be selected (Beun *et al.*, 1999). With this procedure high substrate concentrations are reached in the reactor in short periods of time during the first minutes of the cycle time. The reaction phase comprises, in these cases, two different periods: the feast and famine periods characterised by the presence, or absence, of organic matter in the liquid media, respectively. During the feast period the organic matter is oxidised and stored inside the bacteria cells as glycogen, lipids or Poly-Hydroxy-Alkanoates (PHA) (van Loosdrecht *et al.*, 1997), while during the famine period the bacteria grow on the stored compounds (Beun *et al.*, 2002a).

With this feeding strategy the selection of the appropriate microorganisms to form granules is achieved. The use of this selection pressure was based initially in the hypothesis of Chudoba *et al.* (1973) who claimed that the filamentous microorganisms are slow-growing microorganisms characterised by maximum specific growth rates and affinity constants lower than the floc-forming microorganisms. This peculiarity makes them gain in the competition with the floc formers when the substrate concentrations in the bulk liquid are low. On the other hand, the floc formers take advantage of high substrate concentrations. However Martins *et al.* (2003) found out that this hypothesis is not always applicable. They proposed a new hypothesis to explain this competition based on diffusion limitations inside the flocs caused by low substrate concentrations in the media. In this case filamentous bacteria which grow in one or two dimensions overtake the floc formers in the access to the substrate. On the other hand, other authors (Bossier and Verstraete, 1996; Liu *et al.*, 2004b) also observed that bacteria become more hydrophobic under periodic feast-famine conditions which facilitate microbial aggregation.

What seems clear is that the presence of large substrate concentrations in the liquid media during the aeration phase helps the formation of granules. This periodic feast-famine regime, which acts as a kind of microbial selection pressure condition, is easily achieved in discontinuous reactors such as the SBR type.

***Hydrodynamic shear force***

The structure of mature aerobic granules is hydrodynamic shear force-related. Tay *et al.* (2001b) 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. Furthermore Tay *et al.* (2004b) observed that low superficial air velocities caused an outbreak of filamentous microorganisms, which gave rise to poorly settling sludge and eventual biomass washout, but high superficial velocities significantly improved the settling characteristics of the biomass granules. Therefore the application of high shear forces favours the formation of more compact and denser aerobic granules together with the stimulated production of extracellular polysaccharides and the microbial activity (Tay *et al.*, 2001b; Liu and Tay, 2002). It is well known that extracellular polysaccharides can mediate both cohesion and adhesion of cells and play a crucial role in maintaining structural integrity in a community of immobilized cells (Liu *et al.*, 2004a). Consequently, the enhanced production of extracellular polysaccharides at high shear forces can make granule structure more compact and stronger.

In most of the discussions about shear and the difficulty of measuring the value of applied shear to a certain system makes the comparison between different systems difficult. In systems where the mixture is achieved by air flow (airlift reactors, bubble columns) shear is related to the Dissolved Oxygen (DO) which is a parameter that has been found to seriously affect the stability of the granules when it is limited (Mosquera-Corral *et al.*, 2005a).

### 1.3.2. Removal of nitrogen with aerobic granular biomass

Due to the sensitivity of nitrifying bacteria to environmental factors as well as their low growth rates, it is difficult to obtain and maintain sufficient nitrifying bacteria in conventional WWTPs to perform the nitrification of the present ammonia. Thus the nitrification process is the limiting step. This fact provokes the necessity of the use of high SRT values and of the large volume of the system. Therefore the development of new processes and technologies is required in which the efficient nitrogen removal is obtained together with the organic matter removal. Aerobic granules are usually produced to aerobically remove organic matter, but once aerobic granules are established inside the reactor the processes of nitrification and denitrification can occur simultaneously inside the system. Evidence shows that the problems encountered in the suspended growth systems for nitrogen removal, such as sludge bulking, large space requirement for the treatment plant installation, washout of nitrifying biomass and large production of waste sludge, would be overcame by developing and applying nitrogen removing granules (Yang *et al.*, 2003).

***Biological processes in aerobic granules***

Regarding their bioengineering functions aerobic granules present in their structure different zones where different environmental conditions exist. These zones are located as concentric layers which enable simultaneous organic matter and nitrogen removal, the latter via by the combined nitrification-denitrification processes (Figure 1.5).

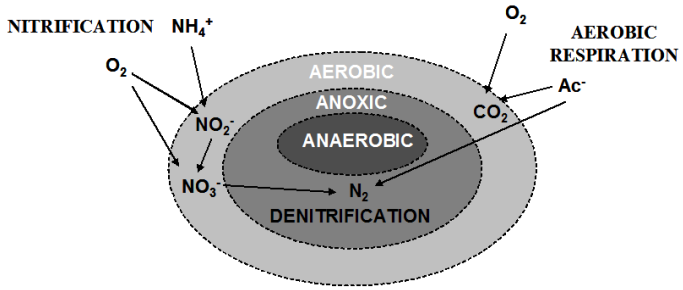


**Figure 1.5.** Biological processes occurring into the aerobic granule (Figueroa *et al.*, 2009).

The way the different processes occur inside the granules varies along the operational cycle since the reactor is sequentially fed and feast and famine periods exist. In the feast period (Figure 1.6a) the concentration of external organic carbon (for example, acetate) is high. This substrate will therefore diffuse into the granules completely. In this case the Dissolved Oxygen (DO) will have a much smaller penetration depth because it will be consumed very rapidly by autotrophic and heterotrophic organisms in the outer layers of the granules. In the feast period DO is used for nitrification, for aerobic conversion of acetate, and for aerobic biomass growth. Since the autotrophic micro-organism need oxygen, they tend to be located where this is available. In the case of granular sludge, this is the outermost layer of the granules. The autotrophic organisms convert ammonia ($NH_4^+$) into nitrogen oxides ($NO_x^-$). The formed $NO_x^-$ will diffuse towards the centre of the granules, but also towards the liquid phase surrounding the granules. In the centre of the granules acetate can be stored anoxically (using $NO_x^-$) as PHB by the heterotrophic organisms. In the famine period (Figure 1.6b) the DO penetration depth is larger than in the feast period since the oxygen concentration in the liquid is higher and the oxygen consumption inside the granule lower. In the centre of the granules $NO_x^-$ is present. The stored PHB in the centre of the granules can be used as organic carbon source for the denitrification. Aerobic conversion of PHB occurs and nitrification as long as there is $NH_4^+$ present.

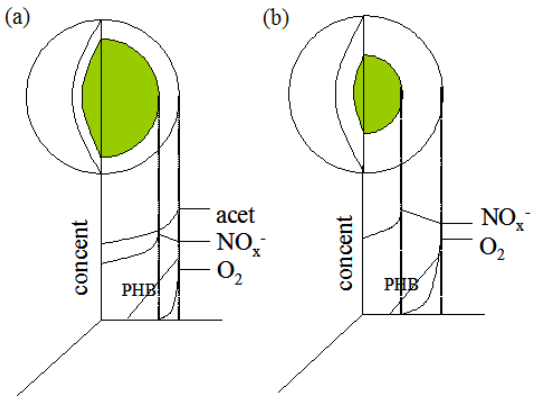


**Figure 1.6.** Schemes of the evolution of the concentration profiles of acetate, PHB, $NO_x^-$and $O_2$ during the feast (a) and the famine (b) period in a SBR (Figueroa *et al.*, 2009).

***Parameters influencing the nitrogen removal***

In the aerobic granular biomass systems simultaneous removal of organic matter and nitrogen compounds implies the coexistence of aerobic heterotrophic, nitrifying and denitrifying populations. Nitrogen removal occurs though the combination of nitrification-denitrification processes inside the aerobic granules, therefore the ratio between aerobic and anoxic volume strongly determines de nitrogen removal efficiency and depends on the size of the aggregate, the DO concentration and the consumption rate of microorganisms (de Kreuk *et al.*, 2007; Li *et al.*, 2008).

***Dissolved oxygen***

Performed experimental work shows that if aerobic conditions are maintained inside the reactor by sufficient aeration, the DO concentration is not a decisive parameter for aerobic granulation, but it has a pronounced effect on the efficiency of the denitrification process. Therefore the amount of oxygen supply has to be optimised in order to minimise the energy consumption in full-scale aerobic systems and to promote nitrogen removal via denitrification, because when all ammonia is oxidized, oxygen diffuses to the core of the granule inhibiting the denitrification process. In order to optimize the process, anoxic phases can be implemented in the SBR cycle configuration (de Kreuk *et al.*, 2007) or the DO concentration could be reduced (Mosquera-Corral *et al.*, 2005a), leading to a more efficient overall N removal.

Chen *et al.* (2011) and Yang *et al.* (2003) studied the application of an anoxic phase placed before and after the aerobic one, respectively, obtaining good results in the nitrogen removal efficiency, although the last option (anoxic phase after aerobic one) implied the addition of an external source of organic carbon. With a similar cycle distribution Quin *et al.* (2005) studied the feasibility of denitrification using the storage compounds like the PHB without addition of an external organic carbon source and observed that the potential role of PHB for denitrification by microbial granules was very limited, being necessary to add an external source of carbon to achieve a complete denitrification.

Respect to the decreasing of the DO concentration along the whole operation Beun *et al.* (2001) predicted, through a simulation model, that the optimal DO value is expected to be around 40% air saturation for adequate nitrogen removal in aerobic granular systems, but this was not experimentally verified. Later, Mosquera-Corral *et al.* (2005a) attempted to study experimentally this fact and revealed the significant role of oxygen on the simultaneous nitrification and denitrification processes. These authors studied the short- and long-term effects of decreased oxygen concentrations on the aerobic granular system and observed that a short-term oxygen reduction did not influence the organic substrate uptake rate and nitrogen removal was favoured by decreased oxygen concentrations, while long-term oxygen reduction (40% saturation level), provoked an increase of the nitrogen removal but also the beginning of the granules disintegration and the biomass washout.

***Granule size***

The size of the aggregates is closely related to the DO concentration profile inside the granules. Li *et al.* (2008) observed that DO only partially penetrated through 500 μm from the granule surface under substrate sufficient condition (feast period) while aerobic condition could be maintained in the entire aerobic granules with a radius less than 2.2 mm under substrate-free condition (famine period). This implies that the size of the granules and the famine-feast regime influences the DO concentration inside the aggregates and therefore the denitrification process in the aerobic granular system. De Kreuk *et al.* (2007) observed that the optimum granule diameter for maximum N and P removal in the standard case operating conditions (DO 2 mg/L, 20 ºC) was found between 1.2 and 1.4 mm.

***COD/N ratio***

The presence of organic matter can also affect the nitrification (Ballinger *et al.*, 2002). Mosquera-Corral *et al.* (2005b) operated an aerobic granular SBR to study the removal of nitrogen related to the organic matter to nitrogen (COD/N) ratio in the feeding media and observed that the granules exhibited different denitrifying activities depending on the COD/N ratio. If the carbon source was not high enough, low COD/N ratios, the denitrification process was not completed and nitrite was produced as intermediate. The effect of COD/N ratio (from 3.3 to 20 g/g) on the formation of aerobic granules was also studied by Yang *et al.* (2005). These authors observed that COD/N ratios in the range studied had no significant impact on the formation of aerobic granules; however had remarkable effects on the activity distribution of heterotrophic, ammonium and nitrite oxidizing bacteria inside the granules. The nitrifying activity increased around 10% with the decrease of the COD/N ratio from 20 to 3.3 g/g, while the heterotrophic activity experienced a reduction. At the lowest COD/N ratio heterotrophic biomass became much less dominant, whereas nitrifying populations would be able to compete with them, and became an important component of the aerobic granules.

***Temperature***

The effect of temperature changes on the conversion processes and the stability of AGS were studied by de Kreuk *et al.* (2005c). These authors observed that the denitrifying capacity of the granules decreased at low temperatures (8 ºC), resulting in an overall poorer nitrogen removal capacity. Nevertheless the temperature dependency of the nitrification process was around 10% lower for aerobic granules than usually found for activated sludge.

### 1.3.3. Applications of aerobic granular technology

An examination of the applications of aerobic granules for wastewater treatment showed many advantages compared to CAS process: excellent settleability, high and stable rates of metabolic activity, resilience to shocks and toxins due to the protection by a matrix of Extracellular Polymeric Substances (EPS), long biomass residence time, biomass immobilization inside the aggregates and the possibility for bioaugmentation (Adav *et al.*, 2008). So although the research in the last years was focused on laboratory scale it demonstrated that this technology has a high potential to treat very different types of wastewater.

The first works were focused in the treatment of synthetic wastewater and a wide variety of substrates were used to obtain aerobic granular biomass including: acetate (Dangcong *et al.*, 1999; Tay *et al.*, 2002b; Lin *et al.*, 2003); ethanol (Beun *et al.*, 1999; Liu *et al.*, 2003c; Yang *et al.*, 2004); glucose, peptone and meat extract (Yi *et al.*, 2003). Tay *et al.* (2001a) studied the difference between granules fed with glucose and acetate and concluded that the granule microstructure and microbial diversity of the granules were related to the type of carbon source. The aerobic granular technology was also used to treat a wide variety of industrial wastewater and toxic compounds and more recently is focused on the treatment of urban wastewater.

***Industrial wastewater***

Different types of industrial wastewater were treated with aerobic granular systems (Table 1.1), which indicate 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 Chemical Oxygen Demand (COD) and nutrients (nitrogen and phosphorus) removal. 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%). The physical properties of the aerobic granular biomass showed values of SVI lower than 75 mL/g TSS, densities larger than 10 g $VSS/L_{granule}$ and mean average diameters (D) that ranged between 1.0 and 6.0 mm. 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).

The treatment of wastewater with toxic compounds is also possible with aerobic granular biomass since the layered structures seen in aerobic granules (Tay *et al.*, 2002a; Li *et al.*, 2008) create concentration gradients and these protect microorganisms from the impact of direct acute toxicity associated with these compounds (Maszenan *et al.*, 2011). Furthermore aerobic granules played a promising role in adsorption of toxic chemicals due to a high surface area, porosity and good settling capability (Adav *et al.*, 2008). Previous research works indicate the feasibility of the application of aerobic granular biomass to the removal of different toxic compounds like: phenol, pyridine, heavy metals, dyes and Endocrine Disrupter Compounds (EDCs).

Aerobic granules have been found to be less susceptible to phenol inhibition due to the compact and dense granule structure, which then served as a phenol diffusion barrier producing lower local phenol concentrations on cells than the bulk value (Liu and Tay, 2004). The compact structure of the granules also retained the phenol degraders and protected the microbial population in the granules core from the inhibitory effect of phenol (Jiang *et al.*, 2002; Tay *et al.*, 2004a; Liu *et al.*, 2009; Ho *et al.*, 2010). Furthermore to degrade phenol with aerobic granular biomass is possible to degrade different types of its derivates like p-nitrophenol, chlorinated phenols and phentachlorophenol (Maszenan *et al.*, 2011). Also aerobic granules cultivated with phenol as co-substrate can biodegrade pyridine (Adav *et al.*, 2007).

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

| Type of wastewater | $OLR_{max}$ (kg COD/ $m^3$·d) | NLR (kg N/ $m^3$·d) | $COD_{removed}$ (%) | $N_{removed}$ (%) | SVI (mL/g TSS) | D (mm) | Ref.[a] |
|---|---|---|---|---|---|---|---|
| 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] |
| Papermaking | - | - | - | - | 75 | 3-6 | [7] |
| Metal-refinery process | - | 1.0 | - | 95 | - | 1.1 | [8] |
| Brewery | 3.5 | 0.24 | 88.7 | 88.9 | 32 | 2-7 | [9] |
| Fish canning | 1.7 | 0.18 | 95 | 40 | 30 | 3.4 | [10] |
| Abattoir | 2.7 | 0.43 | 85 | 93 | - | 0.7-1.6 | [11] |
| Winery | 6.0 | 0.01 | 95 | - | - | 2.0 | [12] |
| Newsprint effluent | - | - | 92 | - | 39 | 1-2 | [13] |
| Pig farm | 4.4 | 0.83 | 87 | 70 | 32 | 5 | [14] |
| Palm oil mill | 6.0 | - | 90 | - | 30 | 0.9 | [15] |
| Abattoir | 1.8 | 0.4 | 99 | 90 | - | < 1.5 | [16] |
| Petrochemical pant | 2.0 | 0.16 | 64 | 30 | 40 | 1.1 | [17] |

[a] [1] Arrojo *et al.* (2004); [2] Schwarzenbeck *et al.* (2004); [3] Cassidy and Belia (2005); [4] Inizan *et al.* (2005); [5] Schwarzenbeck *et al.* (2005); [6] Su and Yu (2005); [7] Hailei *et al.* (2006); [8] Tsuneda *et al.* (2006); [9] Wang *et al.* (2007); [10] Figueroa *et al.* (2008); [11] Yilmaz *et al.* (2008); [12] Lopez-Palau *et al.* (2009); [13] Liu *et al.* (2010a); [14] Figueroa *et al.* (2011); [15] Gobi *et al.* (2011); [16] Pijuan *et al.* (2011) ; [17] Zhang *et al.* (2011).

Due to the settling capability, high surface area and porosity of aerobic granules they can be a good alternative to remove heavy metals and dyes by bioaccumulation or adsorption, being the maximum adsorption density of aerobic granules three times greater than that of sludge flocs (Adav *et al.*, 2008). The removal by biosorption of heavy metals as $Cd^{+2}$, $Co^{+2}$, $Cu^{+2}$, $Ni^{+2}$and $Zn^{+2}$ (Liu *et al.*, 2003b; Zhang *et al.*, 2005; Sun *et al.*, 2008a; Xu and Liu, 2008; Yao *et al.*, 2009) and dyes as Malachite Green, Eriochrome Black T, Reactive Brilliant Blue, Congo Red and Reactive Brilliant Red (Sun *et al.*, 2008b; Hailei *et al.*, 2010; Gao *et al.*, 2011) has been successfully obtained with aerobic granular biomass. Respect to the EDCs, Balest *et al.* (2008) compared the performance of an aerobic granular system and activated sludge process for the removal of five different EDCs obtaining for all the compounds a better removal efficiency with aerobic granular biomass.

***Municipal wastewater***

The development of aerobic granular biomass was successfully obtained with synthetic media to simulate an urban wastewater (Liu *et al.*, 2007; Coma *et al.*, 2010) and also with real urban wastewater (de Kreuk and van Loosdrecht, 2006; Ni *et al.*, 2009; Liu *et al.*, 2010b; Coma *et al.*, 2012). However AGS development with low strength wastewater (such as domestic) requires long start-up periods when organic loads are lower than 1 kg COD/$m^3$·d. Short cycle times and concentrated wastewater must be applied to form granules with low strength wastewater (de Kreuk and van Loosdrecht, 2006). The volume exchange ratio and the settling time of the SBR were found to be two key factors in the granulation with this type of wastewater (Ni *et al.*, 2009).

### 1.3.4. Full scale research: from laboratory to pilot plant

Although the aerobic granular system is a recent technology there are some works at pilot scale which demonstrate that this is a promising alternative for the treatment of wastewater from different sources. It is remarkable that the research works were not only focused on the operation of aerobic reactors but also on some aspects like the way to obtain a quickly start-up or to evaluate economical aspects.

The first pilot research project using the aerobic granular technology was built and operated in The Netherlands with the Nereda™ system (Figure 1.7.A) in order to demonstrate the applicability of the AGS 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. Several operational philosophies were tested to know the conditions which lead to the granulation with municipal wastewater as a substrate. Fast formation of granules was observed under conditions of extensive COD removal, extensive biological phosphate removal and low nitrate effluent concentrations. The potential of the technology is very promising since complete granulation with municipal wastewater as substrate was shown and the extensive nutrient removal seems well feasible. Based in this Nereda™ system a full scale plant with three reactors of 4,500 $m^3$ (height of 9 m and a diameter of 25 m) started to operate in May 2011 in a WWTP of The Nerthelands to treat 1,500 $m^3$/h and 59,000 p.e. including wastewater from slaughterhouses (*Personal communication*).

The feasibility of a full scale AGS technology in comparison with the CAS was studied by de de Bruin *et al.* (2004). Based on total annual costs, a Granular sludge Sequencing Batch Reactor (GSBR) with pre-treatment and a GSBR with post-treatment proves to be more attractive than the reference activated sludge alternatives (6–16%). A sensitivity analysis shows that the GSBR technology is less sensitive to land price and more sensitive to rain water flow. Because of the high allowable volumetric load the footprint of the GSBR variants is only 25% compared to the references.

With a wastewater composed of 40% of domestic and 60% of industrial wastewater Liu et al. (2010b) operated a pilot-scale SBR (32 L) using CAS as inoculum. Compared with the 1 or 2 months required in the case of a lab-scale reactor to achieve aerobic granulation, these authors needed about 400 days to obtain granule-dominant sludge in the pilot-scale SBR. A SVI around 30 mL/g TS and COD and $NH_4^+$-N removal efficiencies above 80 and 98%, respectively, were obtained. In order to reduce the time needed for the start-up of this kind of systems it is possible to apply a similar strategy to that applied in anaerobic reactors that means: the use of pre-cultured granules seeded into the reactor (Liu *et al.*, 2005a; Tay *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).

(A) (B)

**Figure 1.7.** Pictures of different aerobic granular pilot plant: (A) Nereda™ (The Nerthelands) and (B) GSBR University of Santiago (Spain).

With a SBR of 40 L Inizan *et al.* (2005) studied the aerobic granulation with two different substrates (synthetic and pharmaceutical wastewater) obtaining granules with high densities (50-100 g SS/L) and settling velocities (50-100 m/h). For both types of wastewater good dissolved COD removal was observed: 95% and 80% at a load of 7–8 kg COD/$m^3$·d and 5.5 kg COD/$m^3$·d for synthetic and industrial wastewater, respectively.

Ni *et al.* (2009) worked with a reactor of 1 $m^3$ (internal diameter of 0.5 m and height of 6 m) to treat low strength wastewater (<200 mg COD/L). They reached a biomass concentration of 9.5 g VSS/L of which approximate 85% was granular sludge with a diameter between 0.2–0.8 mm and a settling velocity of 18–40 m/h. The average total COD and ammonia removal efficiencies were 90% and 95%, respectively.

From the basis of the AGS 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 WWTP (Di Iaconi *et al.*, 2008; Di Iaconi *et al.*, 2009; Di Iaconi *et al.*, 2010).

Recently Jungles *et al.* (2011) operated a pilot-scale SBR of 100 L (Figure 1.7.B) to obtain AGS with a synthetic wastewater. These authors enhanced the formation of aerobic granular sludge by the selective pressure created by means of decreasing settling time and increasing OLR. The granules had an average diameter around 3.5 mm. The reactor treated OLRs varying between 2.5 and 6.0 kg COD/$m^3$·d reaching removal efficiencies around 96%, which demonstrates the high activity and the ability of the system to withstand high OLR. Nevertheless, a rapid increase in OLR produced a loss of biomass in the reactor due to breakage of the granules.

## 1.4. ANAEOROBIC SLUDGE DIGESTION

As it was previously mentioned the anaerobic digestion is the most widely used process to reduce both primary and biological excess sludge. It is preferred especially in larger WWTPs where the quantity of biogas produced is sufficiently high to be exploited in the same installation with acceptable costs. The anaerobic sludge digestion is based on the fermentation of the organic material by bacteria in the absence of DO and it is applied to thickened sludge in order to reduce, stabilise and partially disinfect the treated volume of sludge.

Due to the recent development of the aerobic granular systems at the moment there are not studies about the anaerobic digestion feasibility of this type of sludge. Although the similar nature of this kind of biomass with respect to the WAS indicates that the anaerobic digestion could be a good option to treat it.

### 1.4.1. Advantages and drawbacks of anaerobic sludge digestion

The anaerobic digestion is a favoured stabilisation method compared to other methods, such as aerobic digestion, due to its ability of transforming organic matter into biogas, reducing the amount of final sludge solids for disposal, destroying most of the pathogens present in the sludge and limiting possible odour problems associated with residual putrid matter (Appels *et al.*, 2008).

- *Biogas production*. The sewage sludge is stabilised to an innocuous and more easily dewatered substance and the energy is produced in the form of biogas, the main constituents of which are methane (60–70 %) and carbon dioxide (30-40%). The process is a net energy producer in most treatment plants in which anaerobic sludge digestion is used. The energy produced is superior to that required to maintain the temperature of the sludge digesters and to meet the energy requirements for mixing. The surplus energy can be used to generate electricity and/or heat.

- *Reduction in the mass and volume of sludge*. The net reduction in the quantity of solids and the volume of sludge requiring disposal are also realised. The destruction of solids is usually about 25-50% of the fed sludge solids and can result in the reduction of the sludge disposal cost.

- *Stabilisation*. The stabilised product of anaerobic digestion is an innocuous sludge with a markedly reduced odour and it can be stored without putrefaction. It contains nitrogen and phosphorus compounds and other nutrients as well as organic material that can be used as a soil conditioner or fertiliser. The pathogen level, the volatile solid content and the concentration of heavy metals are the principal concerns in considering its land disposal.

- *Hygienisation*. A significant inactivation of many pathogenic microorganisms is also accomplished during anaerobic digestion.

For these reasons, anaerobic sludge digestion optimises the operational costs and is considered a major and essential part of a modern WWTP (Appels *et al.*, 2008). However one of its main disadvantages is the relatively high investment costs, since large covered tanks, pumps for feeding and circulating sludge, heat exchangers and compressors for gas mixing are required. Furthermore long SRTs (> 10 days) are required to develop and maintain methane producing bacteria, which implies long start-up. Another drawback is that the supernatant obtained from anaerobic sludge contains suspended solids, dissolved and particulate organic materials, nitrogen and phosphorus and other compounds. When this supernatant is returned to the wastewater treatment it increases the load of the solids, oxygen demand and nutrients that need to be treated.

### 1.4.2. Anaerobic digestion process

The transformation of complex macromolecules, present in sewage, into biogas requires the mediation of several groups of microorganisms. Different steps are necessary for the anaerobic digestion of proteins, carbohydrates and lipids. Four different phases can be distinguished in the overall conversion process (Figure 1.8): hydrolysis, where complex particulate matter is converted into dissolved compounds with a lower molecular weight; acidogenesis and acetogenesis, where the smaller compounds previously generated are converted into acidic compounds, and methanogenesis, where the gasification takes place generating carbon dioxide and methane (van Haandel and Lettinga, 1994). The rate-limiting step in sludge digestion is generally the hydrolysis of particulate organic matter to the soluble substances, due to the fact that most of the organics present in sewage sludge are enveloped by poor biodegradable cell walls and extracellular biopolymers.

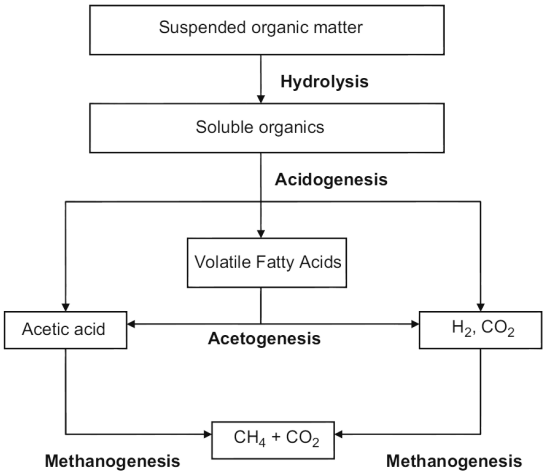


**Figure 1.8.** Reaction sequence for the anaerobic digestion process.

A stable digester operation requires that all the bacterial groups that take part in the anaerobic digestion, are in dynamic equilibrium. Changes in environmental conditions, such as temperature variations or shock loadings of substrate, can affect this equilibrium and result in a build-up of intermediates, such as long chain fatty acids and hydrogen, which inhibit the overall process. Important environmental factors that affect the anaerobic sludge digestion are: temperature, pH, volatile acids and toxic compounds in the influent. The optimum conditions for the maximum methane production during the anaerobic digestion, together with the range of extreme conditions are listed in Table 1.2 (Dohányos and Zábranská, 2001).

**Table 1.2.** Optimum and extreme operational conditions for anaerobic sludge digestion.

| **Variable** | **Optimum** | **Extreme** |
|---|---|---|
| pH | 6.8-7.4 | 6.3-7.9 |
| Oxidation reduction potential (mV) | -520 to -530 | -490 to -550 |
| Volatile acids (mmol/L) | 0.8-8.0 | >35.0 |
| Alkalinity (mg/L as $CaCO_3$) | 1,300-3,000 | 1,000-5,000 |
| OLR (as volatile solids) | | |
| Mesophilic (kg COD/$m^3$·d) | 0.8-2.0 | 0.4-6.4 |
| Thermophilic (kg COD/$m^3$·d) | 1.5-5.0 | 1.0-7.5 |
| Temperature | | |
| Mesophilic (ºC) | 32-37 | 20-42 |
| Thermophilic (ºC) | 50-56 | 45-65 |
| HRT (days) | 12-18 | 7-30 |
| Biogas composition | | |
| Methane (% vol) | 65-70 | 60-75 |
| Carbon dioxide (% vol) | 30-35 | 25-40 |

### 1.4.3. Microbiology of anaerobic digestion

In the anaerobic digestion a series of reactions are performed by various symbiotic microorganisms, which are broadly divided into two groups: acidogens and methanogens (Speece, 1996). The acidogens is the bacterial group that hydrolyzes and ferments organic materials in the wastewater to produce various organic acids and alcohols, and the methanogens is the archaeal group that produces methane using the acidogenic products (Ueno *et al.*, 2001). Thus, complete bioconversion of organic compounds to methane under anaerobic conditions is dependent on the concerted activities of these microbial populations. Moreover, although the anaerobic digestion has been applied successfully to the sludge treatment over the last 100 years, the diversities and distributions of these functional groups and their contributions to the overall anaerobic digesting processes are still poorly understood and molecular methods have only been applied to the analysis of communities in anaerobic digesters since the late 1990's (Godon *et al.*, 1997; Sekiguchi *et al.*, 1998). A better understanding of diversities and population sizes of those functional microbial communities is required to enhance the performance and stability of anaerobic sludge digester (Ariesyady *et al.*, 2007). Factors like the conditions of operation and the type of substrate influence strongly the microbial population and its activity in the anaerobic digester.

For the characterization of the microbial diversity methods based on the 16S rRNA approach can be used (Amann *et al.*, 1995). Between them the Fluorescence *in situ* Hybridization (FISH) has been successfully used to characterize the microbial community in a sludge anaerobic digester (Chouari *et al.*, 2005; Ariesyady *et al.*, 2007). According to Ariesyady *et al.* (2007) in a sludge anaerobic digester the 75% of the microbial community is belonged to the domain *Bacteria* and the 25% to the domain *Archaea*. The shares of the different phyla into the major domain can vary, although several works (Chouari *et al.*, 2005; Ariesyady *et al.*, 2007; Rivière *et al.*, 2009) indicated that some phyla are the predominant in the sludge anaerobic digesters (Table 1.3).

The major phylogenetic bacterial phyla are *Cloroflexi*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, that represented between 70 to 90% of the *Bacteria* domain. Other phyla that can be present in a sludge anaerobic digester in a lower proportion are *Synergistetes*, *Actinobacteria* and *Spirochaeta*. *Chloroflexi* seem to be characteristic of wastewater sludge because it was found in activated sludge and in anaerobic digesters treating municipal sludge (Rivière *et al.*, 2009). Furthermore several studies showed their potential role in the degradation of carbohydrates in anaerobic sludge digesters (Kindaichi *et al.*, 2004; Ariesyady *et al.*, 2007). The phyla *Proteobacteria* includes the *Alfa*-, *Beta*-, *Delta*- and *Gamma-proteobacteria* groups, although the most common among them, in sludge anaerobic digesters, are *Beta*- and *Delta-proteobacteria*. *Beta-proteobacteria* are microorganisms involved in the first steps of the anaerobic degradation as the main consumers of propionate, butyrate and acetate and *Delta-proteobacteria* are related to the oxidation of propionate (Ariesyady *et al.*, 2007). *Bacteroidetes* are known to be proteolytic bacteria (Kindaichi *et al.*, 2004) that intervene in the degradation of proteins and are able to ferment amino acids to acetate (Rivière *et al.*, 2009). *Firmicutes* are syntrophic bacteria, which can degrade Volatile Fatty Acids (VFAs) such as butyrate and its

analogues, this degradation produces $H_2$, which is then degraded by hydrogenotrophic methanogens (Rivière *et al.*, 2009).

**Table 1.3.** Shares of the main phylogenetic groups in sludge anaerobic digesters.

| Group | Rivière *et al.* (2009)[a] | Ariesyady *et al.* (2007)[b] | Chouari *et al.* (2005)[c] |
|---|---|---|---|
| *Bacteria* | | | |
| *Chloroflexi* | 32 | 14 | 6 |
| *Proteobacteria* | 18 | 24 | 21 |
| *Bacteroidetes* | 11 | 21 | 54 |
| *Firmicutes* | 9 | 21 | 11 |
| *Synergistetes* | 4 | N.D. | 3 |
| *Actinobacteria* | 2 | 6 | N.D. |
| *Spirochaeta* | 1 | 5 | N.D. |
| *Archaea* | | | |
| *Methanosarcinales* | 51 | 79 | 1 |
| *Arc I* | 36 | N.D. | 67 |
| *Methanomicrobiales* | 10 | 18 | 16 |
| *Methanobacteriales* | 0.2 | 3 | N.D. |
| *Crenarchaeota* | 2 | N.D. | 16 |

N.D.: Not determined

[a] Percentage of clones from a total of 9,890 clones

[b] Percentage of clones from a total of 521 clones

[c] Percentage of clones from a total of 825 clones

The diversity of archaeal domain within anaerobic sludge digester includes *Methanosarcinales*, *Arc I*, *Methanomicrobiales*, *Methanobacteriales* and *Crenarchaeota* (Table 1.3). *Methanosarcinales* are abundant in mesophilic but not in thermophilic anaerobic processes (Chouari *et al.*, 2005), the most common genus in the anaerobic sludge digesters related to this family is the *Methanosaeta*, a well-known acetoclastic methanogen. Respect to the *Arc I* lineage little data are available about its metabolic capabilities. Chouari *et al.* (2005) showed that this group can grow on formate or $H_2/CO_2$ which may indicate a hydrogenotrophic population. The metabolism of this lineage has not been completely explored and this group may be able to degrade other substrates such as acetate, which would mean a competition between *Methanosarcinales* and *Arc I* (Rivière *et al.*, 2009). The hydrogenotrophic methanogenic pathway is also represented by *Methanomicrobiales* and *Methanobacteriales*, but in smaller proportions than *Arc I*.

### 1.4.4. Pre-treatments to improve the anaerobic biodegradability

The anaerobic biodegradability of sludge depends on a number of properties, including the type of sludge (primary or secondary), the level of inert materials coming from the upstream

catchment, temperature, aerobic/anoxic fraction and, in particular, the sludge age (Carrère *et al.*, 2010) being normally the hydrolysis the limiting step.

Because the rate-limiting step is the hydrolysis of suspended organic matter several efforts have been made to apply pre-treatment methods to improve this hydrolysis rate, enhance sludge reduction and increase biogas production. This is of particular importance during the anaerobic treatment of sewage sludge. By means of efficient pre-treatment the substrate can be more accessible to the anaerobic bacteria, optimizing the methanogenic potential of the sludge. The objective is to accelerate the digestion of the treated sludge, increase the degree of degradation decreasing the amount of sludge to be disposed with the consequent improvement of the energetic balance of the digestion process (Dohányos and Zábranská, 2001).

The enhancement of the biodegradability of particular substrates is mainly based on a better accessibility of the substrate to the enzymes. The disintegration and grinding of solid particles present in sludge creates a new surface where the biodegradation takes place and releases bacteria cells content (cell lysis) where active enzymes may be present. There are several pre-treatments to accomplish this (Carrère *et al.*, 2010): thermal, mechanical and chemical.

***Thermal pre-treatment***

The thermal pre-treatment of sludge consists of heating to moderate (< 100 ºC) or high temperatures up to 220 ºC or more, with contact times of minutes or hours at the required pressure. The application of a thermal pre-treatment produces various effects in the sludge such as: breakdown of the sludge structure (disaggregation of biological flocs), high level of sludge solubilisation, lysis of bacterial cells, release of intracellular constituents and bound water. Therefore the water phase of sludge after a thermal pre-treatment is characterized by a high content of dissolved organic compounds. The effects induced by the thermal pre-treatment can be used to increase biogas production in anaerobic digestion, improving dewaterability, pathogen inactivation and reduction of produced sludge (Foladori *et al.*, 2010).

The main parameter for thermal pre-treatment is temperature, whilst the duration of the pre-treatment generally has less influence. For temperatures above 150 ºC the sludge is liquefied and the contact time has little effect compared to the temperature range. Dohányos *et al.* (2004) proposed a very fast thermal pre-treatment at 170 ºC lasting only 1 min to improve by a 49% the biogas production. Conversely, pre-treatments at moderate temperatures, below 100 ºC, require a longer contact time (from some hours to several days) because the main mechanism in such a case is assumed to be enzymatic hydrolysis. For example Gavala *et al.* (2003) applied a pre-treatment at 70 ºC along 7 days that increased by a 26% the methane production.

The increase of methane production has been linked to the sludge COD solubilisation by means of linear correlations (Carrère *et al.*, 2008). However temperatures above 180 ºC have not been found to cause a further appreciable increase of sludge biodegradability in spite of achieving high solubilisation efficiencies. These observations are attributed to the formation of

refractory compounds linked to Maillard reactions, involving carbohydrates and amino acids in the formation of melanoidins, which are difficult or impossible to degrade (Bougrier *et al.*, 2007).

Numerous studies on thermal hydrolysis as pre-treatment to improve anaerobic digestion have been performed, as shown in Table 1.4 (adapted from Carrère *et al.*, 2010). Although the conditions of the pre-treatments vary between the different works most of the studies reported the optimal temperature range between 160-180 ºC and with a time of pre-treatment from 30 to 60 min.

**Table 1.4.** Thermal pre-treatment methods in mesophilic anaerobic digestion.

| Substrate | Pre-treatment conditions | Anaerobic digestion conditions | Results | Ref. [a] |
|---|---|---|---|---|
| Activated sludge | 70 ºC, 7 days | Batch, 37 ºC | Increase 26% of $CH_4$ production | [1] |
| Primary sludge | 50-65 ºC, 2 days | CSTR, HRT:13-14 days, 35 ºC | Increase 48% of $CH_4$ production | [2] |
| Activated sludge | 175 ºC, 30 min | CSTR, HRT: 15 days, 35 ºC | Increase 62% of $CH_4$ production | [3] |
| Primary sludge | 175 ºC, 30 min | CSTR, HRT: 15 days, 35 ºC | No influence | [3] |
| Mixed sludge | 175 ºC, 30 min | CSTR, HRT: 15 days, 35 ºC | Increase 14% of $CH_4$ production | [3] |
| Activated sludge | 175 ºC, 60 min | Batch, 35 ºC | Increase 42% COD conversion to $CH_4$ | [4] |
| Activated sludge | 180 ºC, 60 min | Batch, 37 ºC | Increase 90% of $CH_4$ production | [5] |
| Mixed sludge | 121 ºC, 60 min | CSTR, HRT: 20 days, 36ºC | Increase 20% of biogas production | [6] |
| Activated sludge | 121 ºC, 30 min | Batch, 37 ºC | Increase 32% of biogas production | [7] |
| Activated sludge | 175 ºC, 40 min | Fixed film reactor, HRT: 2.9 days, 37 ºC | 65% TSS reduction | [8] |
| Activated sludge | 170 ºC, 30 min | CSTR, HRT: 20 days, 35 ºC | Increase 51% of $CH_4$ production | [9] |
| Activated sludge (extended aeration) | 160 ºC, 30 min | WWTP 62000 p.e., HRT: 15 days, 35 ºC | 45% TS removal | [10] |

[a] [1] Gavala *et al.* (2003); [2] Ge *et al.* (2010); [3] Haug *et al.* (1978); [4] Stuckey and McCarty (1978); [5] Tanaka *et al.* (1997); [6] Barjenbruch and Kopplow (2003); [7] Kim *et al.* (2003); [8] Graja *et al.* (2005); [9] Bougrier *et al.* (2006); [10] Chauzy *et al.* (2007).

***Mechanical pre-treatment***

The mechanical pre-treatment consists in the disintegration of the sludge by different methods to achieve the instant cell rupture and resulting in the immediate release of intracellular

compounds. Mechanical disintegration can be achieved using different processes. The choice of a suitable technology has to take into account the disintegration efficiency, the specific energy required and the nature of the sludge (particle size, solid content, etc.). Up to now the main used mechanical pre-treatment techniques are (Foladori *et al.*, 2010):

- *Lysis-thickening centrifuge*. Zábranská *et al.* (2006) studied the full-scale application of lysis-thickening centrifuge as pre-treatment before the anaerobic digestion in three different WWTPs and obtained an increment of specific biogas production in the range of 15-26%, also the organic matter in digested sludge significantly decreased to 48-49%.
- *Stirred ball mills.* Kopp *et al.* (1997) obtained an increase in the VS removal between 12-88% and Baier and Schmidheiny (1997) an increase in the biogas production between 10-62% applying this pre-treatment before the anaerobic digestion.
- *High pressure homogenisers*. Onyeche (2004) integrated a high pressure homogenisation pre-treatment with anaerobic digestion and the results at full-scale indicate a reduction of sludge production of 24% with an increase of biogas production of 25%.
- *High pressure jet and collision.* Nah *et al.* (2000) investigated the effect of this mechanical pre-treatment in a pilot plant (2000 L) obtaining a decrease in the anaerobic digester SRT from 13 to 6 days and an enhancement on VS reduction and unit gas production.
- *Ultrasonic disintegration.* Ultrasounds have widely been applied as pre-treatment of anaerobic digestion. The applied specific energies are usually in the range from 1,000 to 16,000 kJ/kg TS and the biogas enhancement ranges from 24% to 140% in batch systems and from 10% to 45% in continuous or semi-continuous systems (Carrère *et al.*, 2010).

***Chemical pre-treatment***

The chemical pre-treatment includes the oxidation and the alkali treatments. The oxidation transforms the organic compounds into more soluble and more biodegradable oxygenated intermediates. The oxidation of the organic matter before the anaerobic digestion was successfully used as pre-treatment with ozone (Weemaes *et al.*, 2000; Yeom *et al.*, 2002), hydrogen peroxide (Valo *et al.*, 2004; Rivero *et al.*, 2006), catalytic wet oxidation (Song *et al.*, 1992) and wet air oxidation (Yang *et al.*, 2010). Also many research works report that sludge solubilisation significantly increases with alkaline pre-treatment and to a lesser extent by acid pre-treatment (Chen *et al.*, 2007). The alkali pre-treatment is normally combined to a thermal pre-treatment (thermo-chemical pre-treatment) and can be performed by using, in order of solubilisation efficacy, NaOH >KOH>$Mg(OH)_2$ and $Ca(OH)_2$ (Kim *et al.*, 2003).

The use of aerobic granular systems as an alternative to the CAS systems has provided a new source of sludge which requires a further post-treatment. Research has to be performed in order to identify the appropriated treatment process to be applied for the AGS treatment.

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# Chapter 2:

# Materials and methods


## Summary

In this chapter, the materials and methods used in the thesis are described. They comprise those used to measure the parameters to characterize the wastewater (organic matter, nitrogenous compounds, solids, pH and dissolved oxygen), the biogas (composition and production) and the sludge, in special the Aerobic Granular Sludge (AGS).

The AGS was characterised by means of parameters such as Sludge Volume Index (SVI), granules density, average particle diameter, elemental composition and the concentrations in the samples of solids, Chemical Oxygen Demand (COD) and Poly-Hydroxy-Alkanoates (PHA).

The descriptions of the general calculations, like biomass production, performed in the different chapters of this thesis are also provided. Other more specific calculations used exclusively in one work of the thesis are described in the corresponding chapter, as well as the corresponding experimental set-ups.

Finally, the Fluorescent *in situ* Hybridization (FISH) technique, applied to the identification of the microbial populations involved in the biological processes, especially on the anaerobic digestion, is also described.

## 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. Nitrogen

In waters and wastewaters the forms of nitrogen of greatest interest are, in order of decreasing oxidation state, nitrate, nitrite, ammonia and organic nitrogen. All these forms, as well as nitrogen gas ($N_2$), are biochemically inter-convertible following the processes of the nitrogen cycle. Organic nitrogen is defined functionally as the organically bound nitrogen in the trinegative oxidation state, but it does not include all organic nitrogen compounds. Analytically, organic nitrogen and ammonia can be determined together and have been referred to as "Total Kjeldahl Nitrogen" (TKN), a term that reflects the technique used in their determination. Total inorganic nitrogen (IN) is the sum of the nitrate and nitrite forms.

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

TKN was determined in a total organic nitrogen analyzer (Rosemount- Dohrmann DN-1900) equipped with a quimioluminiscence detector with two channels. One channel determines the Total Nitrogen (TN), by oxidation at high temperature, and the other determines the Inorganic Nitrogen (IN), by a chemical reduction. TKN is determined as the difference between TN and IN. All the nitrogen present in the sample is catalytically oxidized to nitrous oxide (NO). The process for TN determination occurs in two steps. The first step is a catalytic (Cu as catalyst) oxidation in the combustion tube at 850 °C and with pure oxygen (1 atm) as carrier gas. The second one is the chemical reduction of residual $NO_2$ with $H_2SO_4$ at 80 ºC and catalyzed by $VaCl_3$. For the IN determination, only the second step (chemical reduction) is used. The NO obtained in the two steps is dried and forced to react with $O_3$ producing an unstable excited state $NO_2$*. The change back of this oxide to its fundamental state releases a proton, from which the determination of TN and IN is carried out by quimioluminiscence, using a multiplicator tube. The instrument is calibrated with a certified standard solution ($KNO_3$, 20 mg N/L) using a response factor method.

***Ammonia nitrogen***

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

*Reagents preparation*

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

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

*Determination procedure*

Place 2.5 mL of sample (diluted if necessary to get a maximum concentration of 1 mg $NH_4^+$-N/L) and add 1.0 and 1.5 mL of solution 1 and 2, respectively. After waiting 45 minutes at room temperature, the concentration of $NH_4^+$-N is measured in a spectrophotometer (CECIL-7200) at 635 nm. The quantification is done with a 5-7 points calibration curve in the range of 0-1 mg $NH_4^+$-N/L, using $NH_4Cl$ as standard (Figure 2.1).

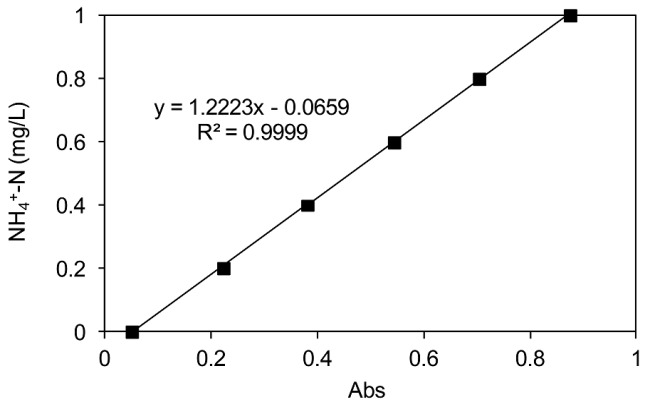


**Figure 2.1.** Calibration curve for ammonia.

***Nitrite***

Nitrite concentration in wastewater is determined following the method 4500-$NO_2^-$-B described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). Nitrite is determined through the formation of a reddish purple azo dye produced at pH 2.0-2.5 by coupling diazotized sulphanilamide with N-(1-napththyl)-ethylenediamine dihydrochloride (NED dihydrochloride).

*Reagents preparation*

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

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

*Determination procedure*

To 5 mL of sample (diluted if necessary to fit the concentration range of the method), 0.1 mL of each solution (sulphanilamide and NED) are added. After waiting 20 minutes for colour stabilisation, the sample is measured in a spectrophotometer (CECIL-7200) at 543 nm. The quantification is done with 6-8 points calibration curve in the range of 0-0.25 mg $NO_2^-$-N/L, using $NaNO_2$ as standard (Figure 2.2).

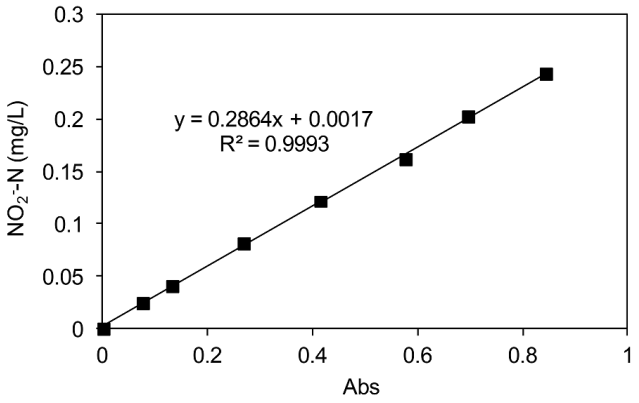


**Figure 2.2.** Calibration curve for nitrite.

***Nitrate***

Nitrate concentration in wastewater is determined following the method 4500-$NO_3^-$-B described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). Measurement of UV absorption at 220 nm enables rapid determination of $NO_3^-$ ions. Because dissolved organic matter also may absorb at 220 nm and $NO_3^-$ does not absorb at 275 nm, a second measurement at 275 nm is used to correct the $NO_3^-$ value.

*Determination procedure*

Place 5 mL of sample (diluted if necessary to get a maximum concentration of $NO_3^-$-N of 2.5 mg/L) and add 0.1 mL of HCl 1 N. Afterwards, the absorbance at 220 and 275 nm is measured in a spectrophotometer (CECIL-7200). The absorbance related to nitrate is obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm. The quantification is done with a 6-8 points calibration curve in the range of 0-3 mg $NO_3^-$-N/L, using $KNO_3$ as standard (Figure 2.3).

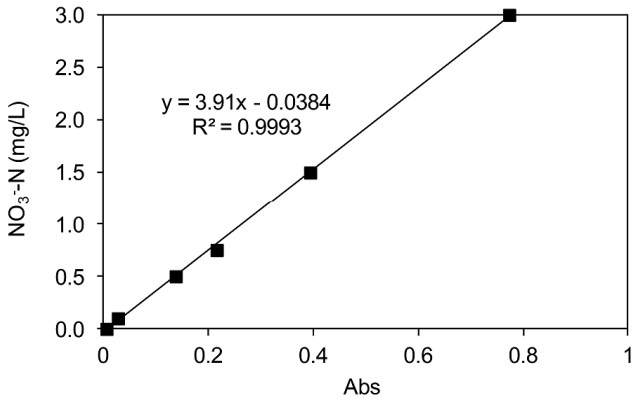


**Figure 2.3.** Calibration curve for nitrate.

### 2.1.2. Inorganic ions

The anions nitrite ($NO_2^-$), nitrate ($NO_3^-$), chloride ($Cl^-$), bromide ($Br^-$), phosphate ($PO_4^{3-}$), sulphate ($SO_4^{2-}$), 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 | | | |

### 2.1.3. 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. From this value the amount of oxidized organic matter is calculated in terms of oxygen equivalents.

The COD of the liquid phase was determined following the method described by Soto *et al.* (1989), which is a modification from the method 5220C (Closed Reflux) of the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The total COD ($COD_T$) was determined using the raw sample and the soluble COD ($COD_S$) using the sample previously filtered through a pore size filter of 0.45 µm (MF-Millipore, Millipore).

***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 ºC for 2 hours, are dissolved in 500 mL of distilled water.
- Ferrous ammonium sulphate titrant (FAS) (0.035 N): 13.72 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ are dissolved in distilled water. Then, 20 mL of concentrated $H_2SO_4$ are added and, finally, the solution is cooled and diluted to 1000 mL.

***Determination procedure***

This procedure is applicable to samples with COD concentrations between 90-900 mg/L. Place 2.5 mL of sample in 10-mL 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 tilted (to avoid mixing). A blank sample using distilled water is prepared in the same way. This blank sample acts as “reference”, providing the COD contain of the distilled water which is supposed to be negligible. 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 /L)

A: volume of FAS solution consumed by the blank (mL)

B: volume of FAS solution consumed by the sample (mL)

### 2.1.4. 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 the COD, the 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-$V_{CSN}$) 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 concentration is determined from the amount of $CO_2$ produced during the combustion of the sample at 680 ºC, using platinum immobilised over alumina spheres as catalyst. The IC concentration is obtained from the $CO_2$ produced in the chemical decomposition of the sample with $H_3PO_4$ (25%) at room temperature. The $CO_2$ produced is optically measured with a non-dispersive infrarred analyzer (NDIR) 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 ($C_8H_5KO_4$) 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.

### 2.1.5. Proteins

The proteins concentration was measured with the Lowry method (Lowry *et al.*, 1951) and expressed in equivalent Bovine Serum Albumin (BSA). 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 colour. Absorbance of the coloured solution is read at a wavelength of 750 nm. The protein concentration is determined from a calibration curve.

***Reagents preparation***

- Solution A: 20 g of $CO_3Na_2$ and 4 g of NaOH are dissolved in 1 L of distilled water. To store at 4 ºC.

- Solution B1: 1 g of $CuSO_4 \cdot 5\ H_2O$ is dissolved in 100 mL of distilled water. To store at 4 ºC.

- Solution B2: 2 g of sodium potassium tartrate is dissolved in 100 mL of distilled water. To store at 4 ºC.

- Solution C: 50, 0.5 and 0.5 mL of solutions A, B1 and B2, respectively, are mixed. This new solution must to be prepared at the moment of the analysis, only can be stored along 24 hours.

- Solution E (Folin and Ciocalteu's phenol reagent): 100 mL of commercial Folin and Ciocalteu's phenol reagent is dissolved in 100 mL of distilled water. To store at 4 ºC in the darkness.

- Standards: 0.2 g of BSA and 1.8 g of NaCl are dissolved in 200 mL of distilled water to obtain a solution of 1 g/L of BSA. Then successive dilutions are applied in order to prepare the Standards for the calibration curve with a concentration of 0, 20, 40, 60, 80 and 100 mg/L of BSA.

***Determination procedure***

Prepare the Sample test tubes appropriately labeled and add 1.0 mL of the sample (diluted if it is necessary). Prepare also a Blank tube adding 1.0 mL of distilled water. Follow the same procedure with the Standards.

Add 3.0 mL of solution C and 0.3 mL of solution E and mix well. Then allow color to develop for 2 hours in the darkness.

Transfer solutions to cuvettes and measure the absorbance of the Standards and Sample tubes versus the Blank at a wavelength of 750 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 (Figure 2.4).

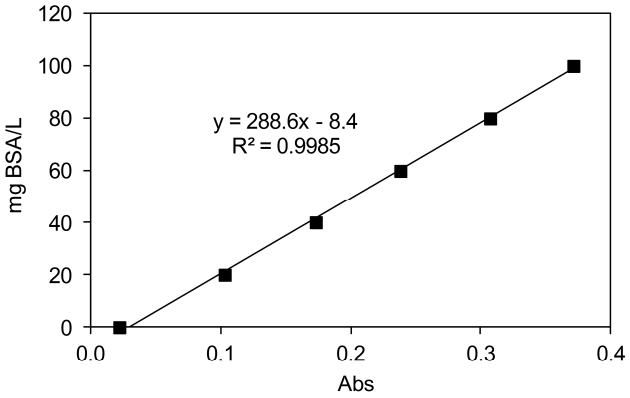


**Figure 2.4.** Calibration curve for proteins.

### 2.1.6. Carbohydrates

The carbohydrates concentration was measured with the anthrone method (Dreywood, 1946) and expressed in equivalent glucose. The carbohydrates react with the anthrone reagent under acidic conditions to yield a blue-green colour. The sample is mixed with sulphuric acid and the anthrone reagent and then boiled until the reaction is completed. The solution is then allowed to cool and its absorbance is measured at 625 nm. There is a linear relationship between the absorbance and the concentration of carbohydrates that are present in the original sample. This method determines both reducing and non-reducing carbohydrates because of the presence of the strongly oxidizing sulphuric acid. It is necessary to prepare a calibration curve using a series of standards of known carbohydrate concentration.

***Reagents preparation***

- Anthrone solution: 200 mg of anthrone are dissolved in 100 mL of sulphuric acid (98%). This solution is not stable and it is necessary to prepare it the same day of the analysis.

- Standards: 0.2 g of glucose is dissolved in 200 mL of distilled water to obtain a solution of 1 g/L. Then successive dilutions are applied in order to prepare the Standards with a concentration of 0, 20, 40, 60, 80 and 100 mg/L of glucose.

***Determination procedure***

Prepare the Sample test tubes appropriately labelled and add 1.0 mL of the sample (diluted if it is necessary), prepare also a Blank tube adding 1.0 mL of distilled water. Follow the same procedure with the Standards.

Add 2.0 mL of the anthrone solution and mix. Then put the test tubes in a water bath at 98 ºC for 10 minutes. To stop the reaction remove the tubes from the bath and cool them immediately with ice.

Transfer solutions to cuvettes and measure the absorbance of the Standards and Sample tubes versus the Blank at a wavelength of 625 nm. Determine the carbohydrate concentration of the Sample tube from the calibration curve prepared from the plot of the absorbance values of the Standards versus their corresponding glucose concentrations (Figure 2.5).

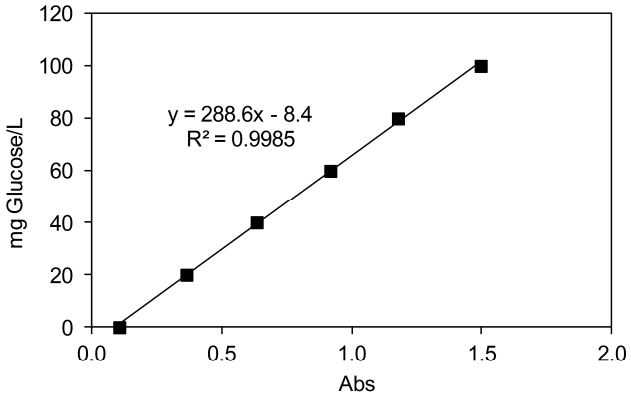


**Figure 2.5.** Calibration curve for carbohydrates.

### 2.1.7. Volatile Fatty Acids (VFA)

The Volatile Fatty Acids (VFA): acetic, propionic, i-butiric, n-butiric, i-valeric and n-valeric, are intermediate products of the anaerobic digestion. A VFA accumulation reflects a kinetic unbalance between the acids producing and the acids consuming microorganisms and it is an indicator of process destabilization.

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

### 2.1.8. Alkalinity

Alkalinity of water samples is defined as the acid-neutralizing capacity. It comprises all the titrable bases, being mainly function of carbonate, bicarbonate and hydroxide content, although it may also include contributions from borates, phosphates, silicates or other bases if they are present.

Hydroxyl ions present in a sample as a result of dissociation or hydrolysis of solutes react with additions of standard acid. Alkalinity thus depends on the end-point pH used. The pH values are suggested as the equivalence points for the corresponding alkalinity concentration as milligrams of $CaCO_3$ per litre.

Total alkalinity (TA) can be considered as the sum of the alkalinity due to bicarbonate plus that corresponding to the VFA and its end-point corresponds to a pH of 4.3. Partial alkalinity (PA), with an end-point pH of 5.75, corresponds to bicarbonate while the intermediate alkalinity (IA), defined as the difference between the TA and the PA, corresponds approximately to the VFA content.

The alkalinity was determined following the method 2320 described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). It consists of a titration of a sample volume (normally 25 mL) at room temperature with standard acid ($H_2SO_4$ standardized against $Na_2CO_3$) to the desired pH, 5.75 for PA and 4.3 for TA. The alkalinity, expressed as mg $CaCO_3$/L, is then calculated from the equation [2.3]:

$$\text{Alkalinity} = \frac{A \times N \times 50000}{V} \qquad [2.3]$$

Where:

A: volume of standard acid used to decrease the pH to 5.75 (PA) or to 4.3 (TA) (mL)

N: normality of standard acid (equivalents/L)

V: sample volume (mL)

### 2.1.9. 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 sensitivity of the instrument is ± 1 mV, corresponding to 0.01 pH units. The electrode is calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

***Dissolved oxygen (DO)***

A dissolved oxygen pocket meter (Oxi 330i, WTW) with a membrane covered galvanic dissolved oxygen sensor (CellOx® 325, WTW) was used to measure the DO concentration in the laboratory scale reactors.

***Conductivity***

Conductivity, expressed in S/cm, was determined with a portable conductivity meter (model 524, Crison).

## 2.2. ANALISYS OF THE GAS PHASE

### 2.2.1. Biogas composition

The biogas composition is an important parameter to determine the amount of methane produced by anaerobic digestion. Besides, it is a good indicator of the reactor performance, since an accumulation of acids in the system normally leads to an increase of the $CO_2$ content in the biogas due to the neutralization of the acids by the bicarbonate.

Biogas composition ($N_2$, $CH_4$, $CO_2$ and $H_2S$) was determined by gas chromatography (HP, 5890 Series II) equipped with a Thermal Conductivity Detector (TCD). The stainless steel column was 2 m long with an external diameter of 1/8" and it was filled with Porapack Q (mesh 80/100). The temperatures of the injector, column and detector were 110, 35 and 110 ºC, respectively. Helium was used as carrier gas with a flow of 15 mL/min. The sample volume (1 mL) was injected through a septum into the entrance of the instrument.

The calibration was performed with a standard mixture of gases ($CH_4$: 66%; $CO_2$: 30%; $N_2$: 2% and $H_2S$: 2%) by a response factor method, using the $CO_2$ as internal standard.

### 2.2.2. Biogas production

The volume of biogas produced by the anaerobic digestion was measured during batch experiments and continuous operation of the anaerobic digester as follows:

***Batch experiments***

The pressure increment in the headspace of the closed bottles was measured with a pressure transducer device (Mano 2000 Leo2 Keller). The biogas production was determined with the measured values of pressure and the volume of the headspace by the application of the ideal gases equation.

***Continuous anaerobic digester***

In the continuous anaerobic digester the biogas production was measured using the flow meter designed by Veiga *et al.* (1990) (Figure 2.6). It consisted of two 20-cm-high glass columns with an internal diameter of 3 cm. The lower ends of the columns are directly connected by means of a tube of 1 cm of diameter and their central regions are connected by a hydraulic valve (a J-tube with an internal diameter of 0.5 cm, the long arm emerging from Column II and the short arm from Column I). The columns contained liquid which fills the columns to an initial level slightly below a level half-way between the two mouths of the J-tube, and which was displaced by gas from the digester entering the top of Column II. Two stainless-steel electrodes at different heights in Column I are connected in series with an electromechanical pulse counter (F.M. Mod. CI851) that counts one unit, every time the liquid in Column I goes up and connects the two electrodes. Once the maximum liquid level is reached it returns to Column II through the J-tube and liquid is at the same level in both columns. This movement of liquid is caused by the fact that the level of the liquid in Column II falls below the lower mouth of the J-tube, with the result that the gas in Column II is discharged to the environment via Column I, the level of liquid

in Column I falls and the counter circuit is broken. The equipment was calibrated to measure 26 ± 0.5 mL per counter unit.

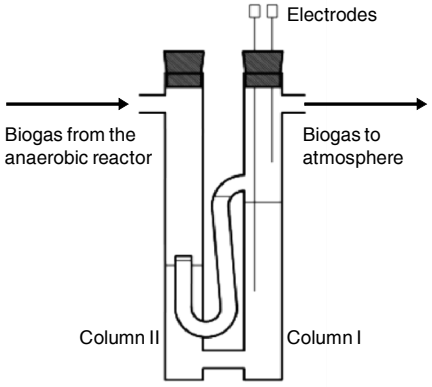


**Figure 2.6.** Biogas flow meter.

## 2.3. BIOMASS CHARACTERISATION

### 2.3.1. Solids

Total Solids (TS), Total Suspended Solids (TSS), Volatile Solids (VS) and Volatile Suspended Solids (VSS) are determined following the methods 2540B, 2540D and 2540E, respectively, described in *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005).

***Determination procedure***

TS are determined weighing a selected well-mixed sample volume (in order to yield a residue between 2.5 and 200 mg) in a previously clean (heated to 103-105 ºC for 2 h) crucible after being evaporated at 103-105 ºC until achieving a constant weight. The increase in weight over that of the empty crucible represents the total solids content in the initial volume of sample. For the determination of TSS, a selected well-mixed sample volume (in order to yield a residue between 2.5 and 200 mg) is filtered through a weighed glassfiber filter (Whatman, GF/C, 4.7 cm of diameter, 1.2 µm of pore size) and the residue retained on the filter is dried to a constant weight (approximately during 2 h) at 103-105 ºC until achieving a constant weight. The increase in weight of the filter represents the TSS content of the sample.

To determine the VS and VSS the residues from methods 2540B and 2540D, respectively, are burnt to constant weight at 550 ºC during half an hour. The weight lost during ignition corresponds to the volatile fraction, since only a small amount of inorganic salts is decomposed and volatilized at that temperature. This determination is useful in the control of the operation of a WWTP because it offers a rough approximation of the amount of organic matter present in the solid fraction of the wastewater and the sludge.

### 2.3.2. Chemical Oxygen Demand (COD)

The basis of the method for the determination of the COD in solid and semi-solid samples (sludge) is the same as that previously described for liquid samples: the oxidation of the organic matter by a strong oxidant like the potassium dichromate in highly acidic solutions by concentrated sulphuric acid. In this case the determination was done following the method 5220B (Open Reflux) of the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005).

***Reagents preparation***

- Digestion solution of potassium dichromate (1 N): 24.52 g of $K_2Cr_2O_7$ are weighed and dissolved in 500 mL of distilled water.
- Sulphuric acid reagent: 9.4 g of $Ag_2SO_4$ are added to 1 L of concentrated $H_2SO_4$ (96%). Complete solution requires two days.
- 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.
- Ferrous ammonium sulphate titrant (FAS) (0.5 N): 196 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ are dissolved in distilled water. Then, 20 mL of concentrated $H_2SO_4$ are added slowly and, finally, the solution is cooled and diluted to 1000 mL.

***Determination procedure***

Place 0.5 g of $HgSO_4$ in each of the Pyrex® glass tubes, which forms insoluble complexes with halogens to avoid that they interfere in the measurement. Add around 1.0-1.5 g of sample (sludge) taking note of the exact weight. Then add 20 mL of distilled water, 10 mL of digestion solution and 30 mL of sulphuric acid reagent slowly on the wall of the Pyrex® tube slightly tilted. A blank sample, using 20 mL of distilled wate,r is prepared in the same way. This blank sample acts as “reference”, corresponding to the COD content of the distilled water. Mix carefully the content of the Pyrex® glass tubes and put the condenser on the top of them. After placing the tubes in the block digester (ECO6 Thermoreactor, Velp Scientifica) 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 10-15 drops of ferroin indicator; the solution is titrated under stirring conditions with the FAS. The FAS solution is standardised daily as follows: 20 mL of distilled water are located into a small beaker, 15 mL of sulphuric acid reagent are added. The mixture is cooled to room temperature and 5 mL of potassium dichromate solution (1 N) are added. To finish 10-15 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 [2.4] and [2.5]:

$$M_{FAS} = \frac{5 \times 1}{V_{FAS}} \qquad [2.4]$$

$$COD = \frac{(A - B) \times M_{FAS} \times 8000}{V_{FAS}} \quad [2.5]$$

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 /g sample)

A: volume of FAS solution consumed by the blank (mL)

B: volume of FAS solution consumed by the sample (mL)

### 2.3.3. Sludge Volume Index (SVI)

The Sludge Volume Index (SVI) was determined according to the procedure specified in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The SVI is the volume in millilitres occupied by 1 g of a suspension after 30 minutes of settling. SVI typically is used to monitor settling characteristics of activated sludge and other biological suspensions. Although SVI is not supported theoretically, experience has shown it to be useful in routine process control. 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 a parameter more representative for granular biomass (de Kreuk *et al.*, 2007). A low $SVI_{30}$ value does not necessarily imply sludge granulation. A granular sludge bed does consolidate very fast, i.e., the terminal SVI is already reached after 10 minutes of settling. The SVI of aerobic granular biomass was determined according to equation [2.6].

$$SVI = \frac{V_{settled\ sludge}}{TSS} \quad [2.6]$$

Where

SVI: Sludge Volume Index (mL/g TSS)

$V_{settled\ sludge}$: volume occupied by the settled sludge after 10 minutes of settling (mL/L)

TSS: Total Suspended Solids concentration (g TSS/L)

### 2.3.4. Granules density

The biomass density (as mass of granules per volume of granules) was determined using the method described by Beun *et al.* (2002). First, a known amount of a homogeneous biomass sample is taken from the reactor and weighed (W1) in a tare weighed 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 ($Ab_1$). This fraction ($Ab_1$) and the original dextran blue solution ($Ab_0$) are analysed by a spectrophotometer at 620 nm. Subsequently the volume occupied by the biomass in the reactor sample is calculated, since dextran blue only diffuses in water and not into the biomass granules. Measuring also the dry weight of the reactor sample (VSS, APHA-AWWA-WPCF, 2005) the density of the granules can be calculated as g of VSS in the biomass per L occupied by the granules (equation [2.7]):

$$\rho_b = VSS \cdot \frac{W_1 - W_2}{W_4 - W_2 - \left( \frac{Ab_0}{Ab_1} \cdot (W_4 - W_3) \right)} \qquad [2.7]$$

Where:

$\rho_b$: density of the granules (g VSS/$L_{granule}$)

VSS: Volatile Suspended Solids concentration of the initial sample (g/L)

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

$W_2$: weight of the empty graduated cylinder (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

### 2.3.5. Average diameter of the granules

The average diameter of the granules in the samples was determined by using a laser radiation technique or by using an image analysis procedure depending on the size of the particles.

***Laser radiation technique***

For particles with a low size (< 1 mm) a laser radiation technique was used (Beckman Coulter LS200 equipped with a LS Variable Speed Fluid Module Plus) to determine the average diameter of the granules. The size analyser has 126 optical detectors and uses reverse Fourier lens optics incorporated in a binocular lens system. This enables the optimisation of the light scattering across the widest dynamic range in a single scan. The range of measure was between 0.4 and 2.0 mm and the time of the analysis between 1-2 minutes.

***Image analysis procedure***

For particles with a high size (> 1 mm) the diameter was followed by using an Image Analysis procedure (Tijhuis *et al.*, 1994). This technique was also used to determine the

morphology of all granules. 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 2.7. 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. Export of the data of interest selected with the software (e.g., area, aspect, roundness, average diameter, etc.) to a worksheet (Excel). 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.

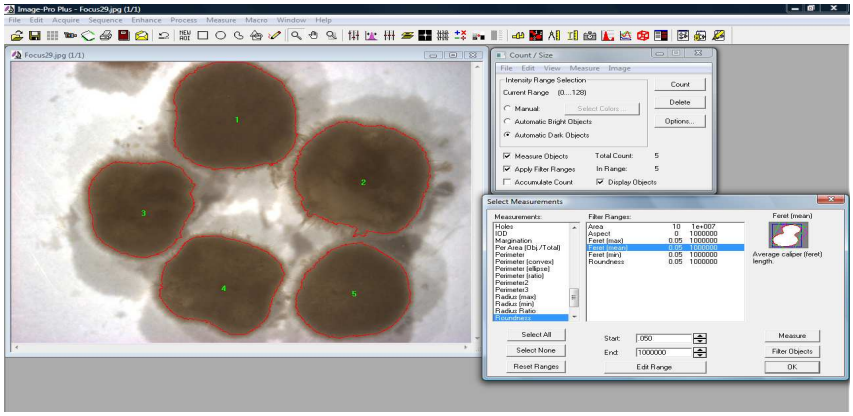

**Figure 2.7.** Original image of a sample of granules and area identified by the software in red once the threshold levels are defined by the user.

4. Utilization of the histogram tool (data analysis tool pack) to calculate the frequency and obtain the histogram. The average diameter can be calculated from the frequency, surface or volume distribution (Figure 2.8).

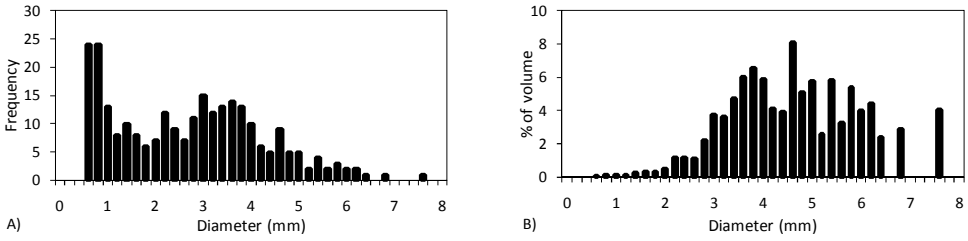


**Figure 2.8**. A) Frequency and B) volume distribution histograms of a granular sludge sample.

### 2.3.6. Scanning Electron Microscopy (SEM)

Morphological studies of the biomass were performed with Scanning Electron Microscopy (SEM) (Digital SEM 440, Leica) controlled by a computer system and provided with a magnification capacity ranging from 15 to 290,000 folds. For SEM analysis the sludge samples were washed three times for 10 minutes with phosphate buffer 0.05 N at a pH value of 7.4, subsequently they were fixed with a solution of glutaraldehyde 3% 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.

### 2.3.7. Elemental analysis

The elemental analysis of the biomass was performed in order to know the composition as $C_nH_aO_bN_cS_d$. First the sample was dried at 105 ºC for 24 hours and then it was crushed to obtain a homogeneous powder. The quantity of sample necessary was between 1-3 mg.

The elemental analysis technique is based on the complete and instantaneous oxidation (combustion) of the sample and the determination of the gases from the combustion through a thermal conductivity detector: model CHNS FISONS EA 1108 (for C, H, N and S) and model CARLO ERBA EA 1108 (for oxygen). The results are expressed as percentage of compound in the sample.

### 2.3.8. Poly-Hydroxy-Alkanoates (PHA)

The Poly-Hydroxy-Alkanoates (PHA) content in the biomass was measured according to a modification of the method of Pijuan *et al.* (2005).

***Reagents preparation***

- Acidified methanol (10% $H_2SO_4$) with internal standard: at 140 mL of pure methanol (99.8%) add 20 mL of $H_2SO_4$ (98%) drop to drop and then complete the volume to 200 mL with pure methanol. Dissolve 20 mg of benzoic acid (internal standard) in this solution.

- Standards: weigh the quantity of standard necessary in Pyrex® tubes by triplicate. The calibration of the method was performed using as standards 3-hydroxybutyric acid and 3-hydroxyvaleric acid copolymer (88:12) (Aldrich) to quantify Poly-Hydroxy-Butyrate (PHB) and Poly-Hydroxy-Valerate (PHV), respectively. Poly-Hydroxy-2-MethylValerate (PH2MV) was quantified using 2-hydroxycaproic acid (98%) (Aldrich).

***Determination procedure***

To stop the biological activity of the biomass sample collected from the reactor it was centrifuged (4700 rpm, during 6 minutes) and the supernatant was removed. The samples were stored in ice until their freeze-drying.

To freeze-dry the tubes containing the samples were covered with Parafilm®, perforated with some small holes in order to avoid that the biomass burst. The samples were inside the freeze-drier for around 24 hours (depending on the volume of the sample) with the following operational conditions: -40 ºC and 0.1 atm.

Then weigh an amount around 30 mg of freeze-dried sludge and place it in Pyrex® tubes by triplicate (take note about the exact quantity of sludge weighed). Add 4 mL of acidified methanol (10% $H_2SO_4$) and 4 mL of chloroform and close the tubes. Digest the samples during 20 h at 100 ºC. The same procedure is required for the standards.

After cooling, free acids must be extracted from the organic phase. In order to extract them add 1 mL of milliQ water and shake the tubes vigorously using the vortex and let them stand until the two phases (organic and aqueous) are separated. Then extract 1 mL of the organic phase that must be filtrated (with glass wool) and dried (with free sodium sulphate) before putting it into the Gas Chromatography (GC) vial.

The analyses were performed in a GC system (Agilent 6850). A volume of 1 µL of the organic phase was injected in 7:1 split mode in a column HP-INNOWAX (30 m × 0.25 mm × 0.25 µm). Results were expressed as weight percentage of PHA in the total biomass.

### 2.3.9. Proteins and carbohydrates

The proteins and carbohydrates concentrations in the sludge samples were measured as was described in the section 2.5.1 for the analysis of the liquid phase, but applying the adequate dilution to ensure that the concentration of these compounds was inside of the calibration range.

### 2.3.10. Lipids

Lipids concentration was measured from biomass samples by accelerated solvent extraction. Dissolved or emulsified oil and grease were extracted by intimate contact with an extraction solvent (petroleum ether).

The biomass sample must be freeze-dried and crushed in a homogenous powder. Then a quantity around 1 g of homogenised sample was exactly weighed and placed in the extraction cell, previously prepared with a cellulose filter and a 1-cm layer of Hydromatrix (dispersant). The sample and Hydromatrix were carefully mixed inside the extraction cell and then the void volume on the top of it was filled with more Hydromatrix. The loaded cell was then mounted in the carousel of a Dionex ASE 200 Accelerated Solvent Extractor (Salt Lake City, Utah, USA) and petroleum ether (40-60º) was used as extraction solvent. The conditions of the extraction were: pressure: 100 bars, temperature: 105 ºC; preheat time: 0 minutes; heat time: 6 minutes; static time: 10 minutes; flushing (fresh solvent added between each cycle as a percentage of the cell volume): 100%; purge time: 100 seconds; number of cycles: 5. The extract (lipids and solvent) was collected in a 60-mL glass vial.

The vials with the extract were placed in a rotary evaporator (Multivapor Buchi) at 47 ºC with $N_2$ to remove the solvent. Then the vials were put in an oven at 105 ºC for 2 hours to ensure the complete solvent evaporation and after cooling they were weighed. The amount of lipids in the sample is the gain in weight of the tare vials.

### 2.3.11. Anaerobic biodegradability tests

The anaerobic biodegradability tests allow determining the ultimate BioMethane Potential (BMP) for different types of substrates, like the sewage sludge. This experiments are important for assessing design, economic and managing issues for the implementation of anaerobic digestion processes.

The tests were carried out in glass flaks of 570 mL (useful volume of 400 mL) with coiled butyl rubber stoppers under the following operational conditions: 35 ºC, 120 rpm, 4 g VS/L of inoculum, 1 g $VS_{substrate}$/g $VS_{inoculum}$, a growth medium containing macro and micro-nutrients (1.8 g/L $NH_4Cl$, 0.7 g/L $KH_2PO_4$, 0.4 g/L $MgCl_2{\cdot}6\ H_2O$, 0.2 g/L $CaCl_2{\cdot}2\ H_2O$, 20 mg/L $FeCl_2{\cdot}4\ H_2O$, 5 mg/L $CoCl_2{\cdot}6\ H_2O$, 1 mg/L $MnCl_2{\cdot}4\ H_2O$, 1 mg/L $NiCl_2{\cdot}6\ H_2O$, 0.5 mg/L $ZnCl_2$, 0.5 mg/L $H_3BO_3$, 0.5 mg/L $Na_2SeO_3$, 0.4 mg/L $CuCl_2{\cdot}2\ H_2O$ and 0.1 mg/L $Na_2MoO_4{\cdot}2\ H_2O$) and 2.6 g/L $NaHCO_3$. Two control tests were carried out under the same conditions: a blank without substrate (only water) to determine the endogenous activity of the inoculum and a test with an easily biodegradable compound (4 g COD/L as ethanol) to check the activity of the inoculum. The volume of biogas produced was determined by the variation of pressure inside the glass flask by means of a pressure transducer (Mano 2000 Leo2 Keller) and its composition by gas chromatography (Micro GC CP-4900 VARIAN).

### 2.3.12. Denitrifying activity

In order to determine the biomass denitrifying capacity on PHA as organic substrate, denitrification tests were performed in batch assays according to the methodology proposed by Buys *et al.* (2000).

Tightly closed vials with a total volume of 38 mL and 25 mL of liquid volume were used to perform the batch assays. The biomass was washed three times with phosphate buffer (0.143 g $KH_2PO_4$/L and 0.747 g $K_2KPO_4$/L) and the concentration in the vial was fixed at around 3.0 g VSS/L. Gas and liquid phases were purged with an inert gas (He) to remove $O_2$. The vials were placed in a thermostatic shaker at 150 rpm and 25 ºC, after some minutes of thermal stabilization the substrate was injected to the vials representing an initial concentration inside the vials of 25 mg $NaNO_3$/L. The production of $N_2$ gas was measured in the gas phase as the increment of pressure in the headspace of the vials with a pressure transducer device (Centrepoint Electronics).

Maximum specific denitrifying activity was estimated from the maximum slope of the curve described by the cumulative $N_2$ production along the time and related to the biomass concentration in the vials.

## 2.4. CALCULATIONS

### 2.4.1. Nitrogen balances: removal, assimilation and denitrification percentages

During the operation of the different used SBRs the estimation of the amounts of nitrogen removed, assimilated and denitrified was performed. The percentage of nitrogen removed ($N_{removed}$) was calculated as the difference between the concentration of all nitrogen compounds ($NH_4^+$, $NO_2^-$ and $NO_3^-$) in the influent and in the effluent of the Sequential Batch Reactor (SBR) and referring this value to the influent concentration. The biological processes taken into account for the nitrogen removal in the operated SBR were nitrogen assimilation for biomass growth and nitrification-denitrification for ammonia removal.

The estimations of the nitrogen assimilated ($N_{assimilated}$) and denitrified ($N_{denitrifed}$) in a SBR were determined according to Mosquera-Corral *et al.* (2005b). 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 ($\Delta W_P$) was estimated from the biomass increase in the reactor and the amount of biomass washed out in the effluent using the equation [2.8]:

$$\Delta W_P = \Delta X_r \cdot V_r + \bar{X}_{eff} \cdot Q \cdot \Delta t \qquad [2.8]$$

Considering a general composition of the biomass as $C_5H_7NO_2$ the average amount of nitrogen assimilated for biomass growth ($\Delta W_N$) was calculated using equation [2.9] as:

$$\Delta W_N = \Delta W_P \cdot \frac{14 \text{ g-mol N}}{113 \text{ g-mol biomass}} \qquad [2.9]$$

The percentages of nitrogen assimilated ($\%N_{assimilated}$) and denitrified ($\%N_{denitrified}$) were calculated using equations [2.10] and [2.11], respectively.

$$\%N_{assimilated} = \frac{\frac{\Delta W_N}{Q \cdot \Delta t}}{N_{influent}} \cdot 100 \qquad [2.10]$$

$$\%N_{denitrified} = \%N_{removed} - \%N_{assimilated} \qquad [2.11]$$

Where

$\Delta W_P$: amount of produced biomass (g VSS)

$\Delta X_r$: change of biomass concentration during each period (g VSS/L)

$V_r$: reactor volume (L)

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

Q: flow rate (L/d)

$\Delta t$: length of the selected period (d)

$\Delta W_N$: average amount of nitrogen assimilated (g N)

$N_{influent}$: concentration of all nitrogen compounds in the influent (g N/L)

$\%N_{removed}$: percentage of nitrogen removed (%)

$\%N_{assimilated}$: percentage of nitrogen assimilated (%)

$\%N_{denitrified}$: percentage of nitrogen denitrified (%)

### 2.4.2. Biomass production yield (Y)

The biomass yield (Y) of the aerobic granules expressed in terms of gram of biomass produced per gram of organic matter removed was calculated for selected operational periods of the used reactors according to equation [2.12].

The obtained amount of produced biomass was calculated according to equation [2.8]. The amount of organic matter removed was calculated from the experimental data obtained from the performance of the reactor in the selected operational period, 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 [2.12]:

$$Y=\frac{\Delta W_p}{(\overline{COD_{inf}} - \overline{COD_{eff}})\cdot Q\cdot \Delta t} \qquad [2.12]$$

Where

Y: biomass yield of the aerobic granules (g VSS /g $COD_{removed}$)

$\Delta W_P$: 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)

$\Delta t$: length of the selected period (d)

### 2.4.3. Determination of the oxygen gas-liquid transfer coefficient ($k_{La}$)

An experimental estimation of the ($k_La$) was carried out by means of a dynamic method. The procedure consisted of registering the increase of DO concentrations in the liquid media of the reactor in the absence of biomass after the reestablishment of the aeration beginning with the concentrations of DO close to zero. The mass balance was applied to oxygen according to equation [2.13], which was integrated to obtain the equation [2.14]. This equation was used to represent the obtained data and to calculate the value of $k_La$ as the slope of the obtained curve.

$$\frac{dC_{O_2}}{dt}=k_La\cdot(C^*_{O_2}-C_{O_2}) \qquad [2.13]$$

$$\ln\left(\frac{C^*_{O_2}-C^{t=0}_{O_2}}{C^*_{O_2}-C_{O_2}}\right)=k_La\cdot t \qquad [2.14]$$

Where:

$k_La$: oxygen gas-liquid transfer coefficient ($d^{-1}$)

$C_{O2}^*$: saturation DO concentration at the experimental temperature (mg/L)

$C_{O2}^{t=0}$: DO concentration at the beginning of the experiment (mg/L)

$C_{O2}$: measured DO concentration in the bulk liquid (mg/L)

t: time (d)

### 2.4.4. Consumption and production rates of the nitrogen compounds

The activities of the different microbial populations present in the SBR were calculated using the concentration profiles obtained during a whole cycle of operation, based on the procedure described by Mosquera-Corral *et al.* (2005a) and according to the representation of Figure 2.9.

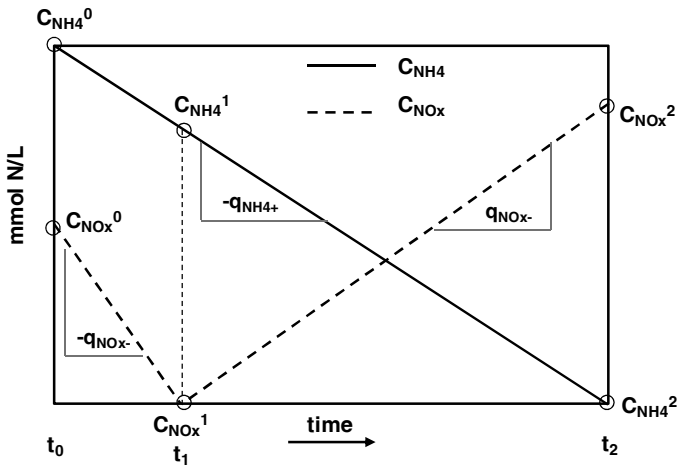


**Figure 2.9**. Schematic representation of the nitrogen compounds concentrations during an operational cycle of a SBR and definition of the calculated parameters.

The specific consumption rates for ammonia ($-q_{NH4+}$) and nitrogen oxides ($-q_{NOx-}$) and the specific production rate of nitrogen oxides ($q_{NOx}$) were calculated using the equations [2.15], [2.16] and [2.17], respectively.

$$-q_{NH_4^+} = \frac{C^1_{NH_4^+} - C^2_{NH_4^+}}{(t_2 - t_1) \cdot C_{biomass}} \quad [2.15]$$

$$-q_{NO_x^-} = \frac{C^0_{NO_x^-} - C^1_{NO_x^-}}{(t_1 - t_0) \cdot C_{biomass}} \quad [2.16]$$

$$q_{NO_x^-} = \frac{C^2_{NO_x^-} - C^1_{NO_x^-}}{(t_2 - t_1) \cdot C_{biomass}} \quad [2.17]$$

Where

$t_0$: time at the beginning of the cycle (h)

$t_1$: time at the end of the feast phase (h)

$t_2$: time at the end of the famine phase (h)

$C_c^t$: concentration of each compound (C) in a certain time (t) (mmol N/L)

$C_{biomass}$: concentration of biomass (mmol C/L)

$q_c$: specific rate of each compound (C) (N-mol/(C-mol·h))

The specific PHB synthesis rate ($q_{PHB}$) and the specific acetate uptake rate ($-q_{Ac^-}$) were calculated in a similar way than the previous parameters and according to Beun *et al.* (2000), assuming that they follow a zero-order kinetic and a constant biomass concentration along a cycle of operation. The concentration of biomass ($C_{biomass}$) as mmol C/L was calculated using the stoichiometric composition of a biomass sample collected from the SBR measured by elemental analysis ($CH_{1.77}N_{0.20}O_{0.59}S_{0.004}$) and the solids concentration.

### 2.4.5. Free ammonia

The concentration of free ammonia ($NH_3$) was calculated at the operational temperature from the $NH_4^+$ concentration and the pH in the bulk liquid using equation [2.18] according to the expression proposed by Anthonisen *et al.* (1976)

$$C_{NH_3} = \frac{C_{NH_4^+}}{\left( \frac{e^{\frac{6344}{T+273}}}{10^{pH}} + 1 \right)} \quad [2.18]$$

### 2.4.6. Solids repartition

The solids repartition as the percentage respect to the total solids for particulate mineral ($P_{mineral}$), particulate organic ($P_{organic}$), soluble mineral ($S_{mineral}$) and soluble organic ($S_{organic}$)

fractions were calculated according to equations [2.19], [2.20], [2.21] and [2.22], respectively, where solids concentrations are expressed as g/L.

$$P_{mineral} = \frac{TSS\text{-}VSS}{TS} \cdot 100 \quad [2.19]$$

$$P_{organic} = \frac{VSS}{TS} \cdot 100 \quad [2.20]$$

$$S_{mineral} = \frac{(TS\text{-}VS)\text{-}(TSS\text{-}VSS)}{TS} \cdot 100 \quad [2.21]$$

$$S_{organic} = \frac{VS\text{-}VSS}{TS} \cdot 100 \quad [2.22]$$

### 2.4.7. Solubilisation

The solubilisation percentage due to the thermal pre-treatment for COD, VS, proteins and carbohydrates, was calculated according to equation [2.23] as the ratio between the soluble fraction after the pre-treatment ($C_S$) minus the initial soluble fraction ($C_{S0}$) and divided by the initial particulate fraction ($C_{P0}$), where C represented the concentration of the compound (COD, VS, proteins or carbohydrates) expressed as g/L.

$$S_C = \frac{C_S - C_{S0}}{C_{P0}} \cdot 100 \quad [2.23]$$

### 2.4.8. BioMethane Potential (BMP) and BioDegradability (BD)

The BMP determined by means of batch anaerobic tests was expressed as the volume of cumulative methane produced at the end of the assay in standard conditions per gram of total COD of substrate fed (N-mL $CH_4$/g $COD_{fed}$) and according to equation [2.24].

$$BMP = \frac{V_{CH4}}{COD_{fed}} \quad [2.24]$$

The volume of cumulative methane produced during the batch anaerobic tests ($V_{CH4}$) was determined by the equation [2.25], measuring the variation of pressure increase inside the glass flasks and the methane composition of the produced biogas.

$$V_{CH4} = \sum \Delta V_{CH4}(j) \quad [2.25]$$

$$\Delta V_{CH4}(j) = \Delta N_{CH4}(j) \cdot \frac{RT_0}{P_0} = \Delta N_{CH4}(j) \cdot \frac{8.314 \times 273.15}{10^5} \cdot 10^6 \quad [2.26]$$

$$\Delta N_{CH4}(j) = \left[ y_{CH4}(j) \cdot P(j) \cdot \frac{V_h}{RT} \right] - \left[ y_{CH4}(j-1) \cdot P_{atm}(j-1) \cdot \frac{V_h}{RT} \right] \quad [2.27]$$

$$P(j) = \Delta P(j) + P_{atm}(j) \quad [2.28]$$

Where

$\Delta V_{CH4}$ (j): volume of methane in standard conditions in the measurement "j" (N-m$L_{CH4}$)

$\Delta N_{CH4}$: number of methane moles in the measurement "j" (mol)

R: ideal gas constant (J/mol·K)

$T_0$: temperature in standard conditions (K)

T: temperature of the experiment (K)

$P_0$: pressure in standard conditions (Pa)

P (j): pressure of the biogas in the measurement "j" (Pa)

$P_{atm}$ (j) and $P_{atm}$ (j-1): atmospheric pressure during the actual measurement "j" and the previous one "j-1" (Pa)

$\Delta$P (j): differential pressure measured inside the glass flask (Pa)

$y_{CH4}$ (j) and $y_{CH4}$ (j-1): volume fraction of methane in the biogas in the actual measurement "j" and in the previous one "j-1"

$V_h$: volume of the head space in the flask ($m^3$)

The percentage of BioDegradability (BD) was determined according to Mottet *et al.* (2010) by dividing the BMP by the maximum theoretical $CH_4$ production in standard conditions (350 N-mL $CH_4$/g $COD_{fed}$ at 1 atm and 0 °C).

$$BD = \frac{BMP}{350} \cdot 100 \quad [2.29]$$

## 2.5. IDENTIFICATION OF BACTERIA POPULATIONS BY FLUORESCENCE IN SITU HYBRIDISATION

The abundance of the different populations of microorganisms present in the sludge samples was studied by the Fluorescent *in situ* Hybridization (FISH) technique. The FISH enables the identification of microorganisms at any desired taxonomical level, depending on the specificity of the used probe. By means of this technique specific regions of the 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 the organism can be later detected microscopically. Hybridization was performed according to the protocol described by Amann *et al.* (1995) with 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 value 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 of milliQ water are heated to 60 °C and 0.4 g of paraformaldehyde are added. One drop of NaOH 1 M is added and the solution is vigorously shaken until complete solubilisation is obtained (1-2 minutes). Then, 3.3 mL of PBS (3x) are added and the pH is adjusted to 7.2 with HCl (one drop HCl 1 M). Finally, the solution is filtered through a 0.2 µm membrane filter.

- Hybridization buffer: The buffer is prepared in 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: This buffer is prepared in a 50 mL Falcon tube by mixing: 1 mL of Tris/HCl (pH 8.0) and required amounts of NaCl 5 M and 0.5 M EDTA (pH 8.0) according to the percentage of formamide used with the applied probe (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 solution 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 cells 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 minutes. Cells are dehydrated by successive passage through 50%, 80% and 98% ethanol (3 minutes each) and dried under air.

***Hybridisation:***

The hybridisation buffer is prepared and kept at room temperature. The hybridisation tube is prepared by placing a folded tissue inside 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 hybridisation 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 the Falcon tube containing the washing buffer and incubated for 15 minutes 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 (which amplifies the fluorescence, avoids fading and contains DAPI dye) and the cover slip is put on the slide.

Specimens can now be analysed with an epifluorescence microscope (Axioskop 2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics). The used acquisition software was RSI image v 1.7.3. (Roper Scientific, Inc.).

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 on the requirements (Figure 2.10). The blend mode "ligthen" was chosen.

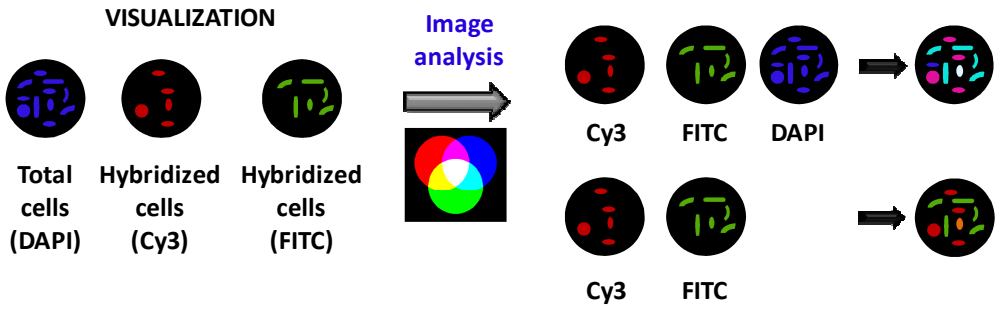


**Figure 2.10.** Schematic representation of the colours resulting after merging the different images corresponding to different dyes.

***FISH probes***

A schematic tree resuming the most important probes applied in this study, indicating the main bacteria detected by each probe, is shown in Figure 2.11.

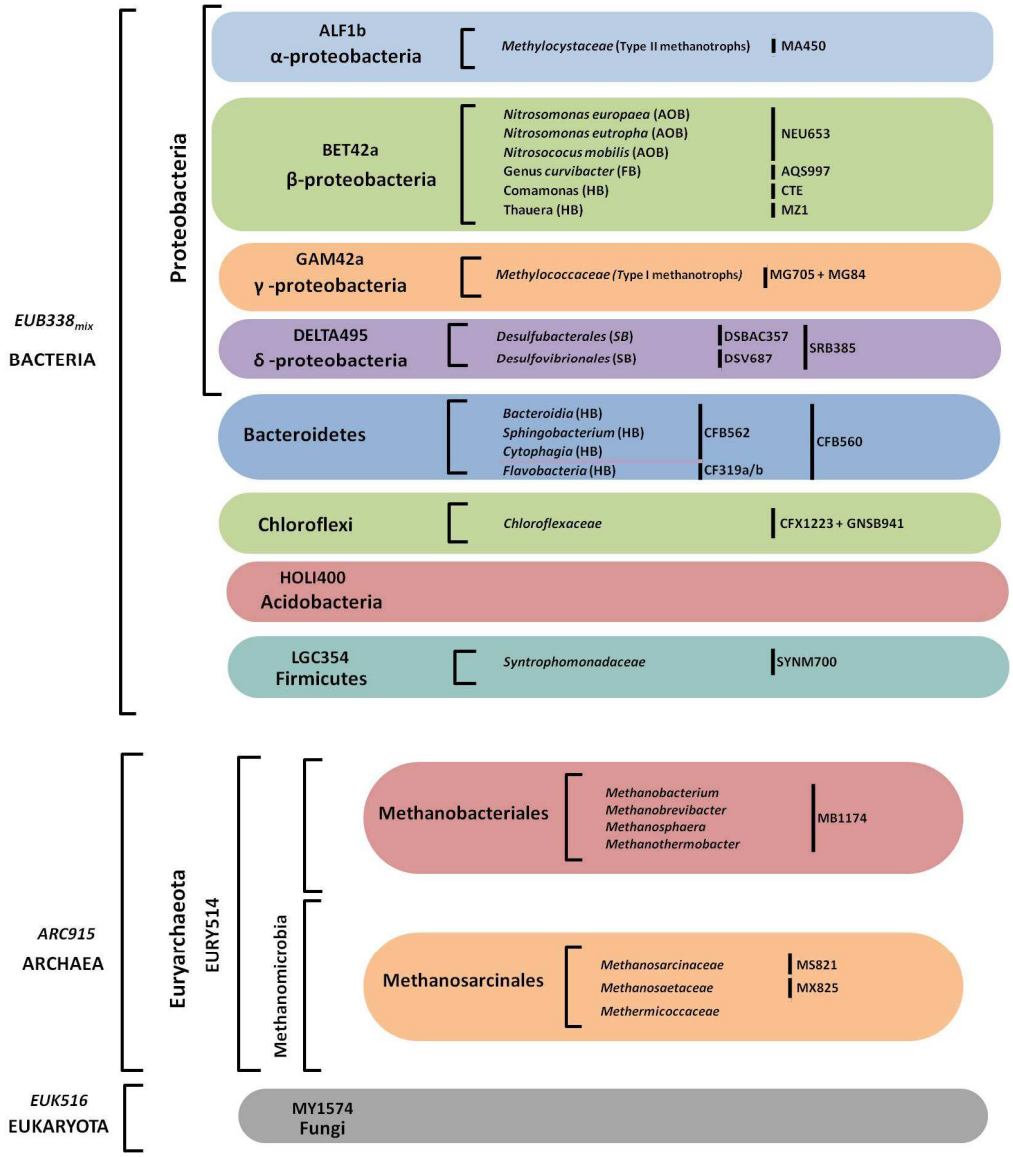


**Figure 2.11.** Different probes for Bacteria, Archaea and Eukaryota superkingdom applied in this thesis (AOB: ammonium-oxidizing bacteria, HB: heterotrophic bacteria, FB: filamentous bacteria and SB: sulphur bacteria).

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 used methods will be mentioned.

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# Chapter 3:

# Stability of aerobic granular biomass treating the effluent from a seafood industry [1, 2]


## Summary

The lower surface requirement of aerobic granular systems in comparison with the Conventional Activated Sludge (CAS) ones makes it a suitable technology to treat industrial effluents, since normally the land available for the wastewater treatment is scarce and expensive for the industries.

In this chapter the effluent from a seafood plant, after a physical-chemical pre-treatment and characterized by a high variable composition, was used to study the development of aerobic granular biomass. In a first stage the reactor treated Organic Loading Rates (OLRs) between 2 and 4 kg $COD_S/m^3 \cdot d$ with a removal efficiency of 90%. At this point although the conditions for nitrogen removal were not optimized, percentages of 65% and 30% for ammonia and total nitrogen removal, respectively, were achieved treating a Nitrogen Loading Rate (NLR) of 0.45 kg $NH_4^+$-$N/m^3 \cdot d$. The developed aerobic granular biomass presented good settling properties: Sludge Volume Index (SVI) of 35 mL/g TSS, density of 60 g $VSS/L_{granule}$ and average diameter of 2.8 mm. In a second stage the reactor was submitted to a high variation in the OLR (from 3 to 13 kg $COD_S/m^3 \cdot d$) to simulate the real conditions of the industry and to study the operation stability of the system. The obtained results showed that the organic matter removal efficiency was not affected but the granules physical properties and the nitrogen removal efficiency experienced a detrimental effect caused by the increase of applied OLR.


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[1] **Val del Río, A.**, Figueroa, M., Arrojo, B., Mosquera-Corral, A., Campos, J.L., García-Torriello, G. and Méndez, R. (2012). Aerobic granular SBR systems applied to the treatment of industrial effluents. *Journal of Environmental Management,* 95(Supplement), S88-S92.

[2] **Val del Río, A.**, Figueroa, M., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2012). Stability of aerobic granular biomass treating the effluent from a seafood industry. *International Journal of Environmental Research*. Accepted.

## 3.1. INTRODUCTION

The uncontrolled discharges of urban and industrial wastewater without treatment suppose an environmental problem. The choice of the adequate treatment is conditioned by many factors, but generally the capital and operational costs are some of the most important ones. For this reason treatment systems are required not only to be able to eliminate the pollution but also to be economically viable. In this sense aerobic granular systems can be an interesting technology because their surface requirement and sludge production are lower than those of the CAS systems (de Bruin *et al.*, 2004; Campos *et al.*, 2009a).

The low footprint of the aerobic granular technology is related to the good settleability of aerobic granules which allows the accumulation of large biomass concentrations inside the system to operate at high loading rates without the necessity of a secondary settler (Beun *et al.*, 1999). Moreover, due to the stratification of microbial populations inside the biomass granules, the simultaneous removal of organic matter, nitrogen and phosphorus can be achieved in a single unit (de Kreuk *et al.*, 2005). In comparison with the CAS systems the yield of the biomass in aerobic granular systems is lower which would also contribute to the decrease of the operating costs (Campos *et al.*, 2009b).

All these advantages make the aerobic granular technology as a good option to treat industrial wastewaters. However this type of effluents is characterized by high loading rates and a variable composition, which could affect the stability of aerobic granules. Different studies showed that the maximum applicable loading rate in an aerobic granular system is limited and depended on the type of substrate. In Table 3.1 a summary of different works with aerobic granular biomass for the treatment of industrial and synthetic wastewaters at high OLRs is presented. In most of the cases when the maximum capacity of the system was reached it led to physical granules instability. The explanation for this instability differs in the literature. Liu and Liu (2006) attributed it to the overgrowth of filamentous microorganisms and Zheng *et al.* (2006) to an intracellular protein hydrolysis and degradation at the anaerobic granule core; Adav *et al.* (2010) demonstrated that under a high OLR the microorganims lose their capability for autoaggregation due to a reduction in the quantity of secreted protein. It seems clear that each type of substrate has a maximum OLR that can be treated in the system without affecting the granule stability. However it would be also necessary to know the recovery capacity of the granular system when an instability episode occurs. Most of the research works (Table 3.1) have been performed to study the effects of progressive increases in the applied loads but the effect of a situation where continuous variations in the influent composition occurs has not been studied. This information is very interesting when aerobic granular systems are applied to the treatment of industrial effluents with high fluctuations of their composition such as those effluents from seafood industry (Vandanjon *et al.*, 2002; Ferjani *et al.*, 2005).

Since the use of a pre-treatment process previously to the biological one, usually involving the addition of reagents such as coagulants and flocculants, is very common to separate fats and solids the possible effects of a residual amount of these reagents on the biomass physical properties and activity should be taken into account (Lees et al., 2001; Ren et al., 2008).

**Table 3.1.** Performance of some aerobic granular reactors treating high OLRs.

| Type of wastewater | Granulation [a] (time-OLR-F/M) | $OLR_{max}$ [b] | NLR [b] | $COD_r$ [c] (%) | $N_r$ [c] (%) | SVI [b] | Diameter (mm) | Granules stability | Ref.[e] |
|---|---|---|---|---|---|---|---|---|---|
| Glucose | 21 – 6.0 – ND | 15.0 | - | 92 | - | 31 | 3.3 | entire | [1] |
| Acetate | 21 – 6.0 – ND | 9.0 | - | 97 | - | 42 | 4.2 | disintegrated | [1] |
| Sucrose | 30 – 6.0 – 1.1 | 6.0 | - | 96 | - | 50 | 10.0 | disintegrated | [2] |
| Acetate | 15 – 16.7 – 2.4 | 21.3 | - | 95 | - | 40 | 4.0 | disintegrated | [3] |
| Dairy products | 21 – 1.0 – 1.0 | 7.0 | 0.70 | 90 | 70 | 60 | 3.5 | entire | [4] |
| Dairy plant | 105 – 4.5 – 1.1 | 5.9 | 0.28 | 90 | 80 | 50 | - | filamentous outgrowth | [5] |
| Soybean-processing | 20 – 6.0 – 0.8 | 6.0 | 0.30 | 98 | - | 26 | 1.2 | entire | [6] |
| Winery [d] | 40 – 2.7 – 0.8 | 6.0 | 0.01 | 95 | - | - | 2.0 | entire | [7] |
| Pig farm | 25 – 2.2 – 0.5 | 7.3 | 0.96 | 91 | - | 72 | 5.2 | filamentous/ disintegrated | [8] |
| Palm oil mill | 110 – 3.0 – 1.1 | 6.0 | ND | 90 | - | 21 | 4.0 | disintegrated | [9] |

ND: not data

[a] Granulation: time (days), OLR (kg COD/$m^3$·d) and F/M (g $COD_S$/g VSS·d).

[b] $OLR_{max}$ (kg COD/$m^3$·d), NLR (kg N/$m^3$·d), SVI (mL/g TSS)

[c] Percentage of COD and N removed.

[d] Granulation with synthetic media.

[e] [1] Moy *et al.* (2002); [2] Zheng *et al.* (2006); [3] Adav *et al.* (2010); [4] Arrojo *et al.* (2004); [5] Schwarzenbeck *et al.* (2005); [6] Su and Yu (2005); [7] Lopez-Palau *et al.* (2009); [8] Figueroa *et al.* (2011); [9] Gobi *et al.* (2011).

## 3.2. OBJECTIVE

The objective of the research work presented in this chapter is to study the feasibility of the use of an aerobic granular system to treat an industrial effluent produced in a seafood industry, characterized by its high variable organic content and presence of residual amounts of coagulant and flocculant reagents from the previous physical-chemical treatment. Special attention will be paid to the physical characteristics of the granular biomass, performance stability and organic matter and nitrogen removal capacities when the reactor is submitted to sudden variations of the applied loading rate.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Experimental set-up

A Sequencing Batch Reactor (SBR) with a total volume of 2.7 L and a working volume of 1.8 L was used (Figure 3.1A). The dimensions of the unit were: height of 480 mm and inner diameter of 85 mm. The H/D ratio was of 5.6. The reactor was operated at room temperature (15-20 ºC) and the Dissolved Oxygen (DO) concentration was between 4 and 8 mg $O_2$/L. The hydraulic retention (HRT) time was kept at 0.25 d. The cycle of operation was of 3 hours distributed as follows: 3 minutes of feeding, 171 minutes of aeration, 3 or 1 minute of settling and 3 or 5 minutes of effluent withdrawal.

A layout of the experimental set-up is shown in Figure 3.1B. Oxygen was supplied to the reactor by means of air spargers to promote the formation of small air bubbles. A set of two peristaltic pumps was used to feed and to discharge the effluent, respectively. The influent was introduced through a port located at the top of the reactor. The effluent was discharged through the sampling port placed at medium height of the column reactor and the exchange volume ratio was fixed at 50%. A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations of the pumps and valves and the length of every phase comprising the cycle.

(A) (B)

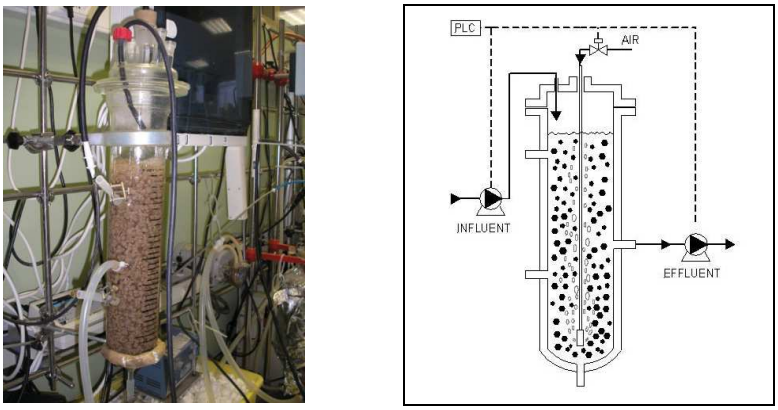


**Figure 3.1.** (A) Image of the SBR and (B) layout of the experimental set-up.

### 3.3.2. Operational strategy

The SBR was fed with the effluent from a seafood industry which was pre-treated in an air floatation unit to remove thick solids and fats by addition of coagulant and flocculant reagents. The wastewater was stored at 4 ºC prior to be fed to the SBR.

The industrial wastewater was characterized by a wide variability of its composition due to the different products processed in the plant (prawn, squid, hake, etc.). Due to this variability the reactor was operated in two different stages (Table 3.2). The Stage I (days 0-295) corresponded to the start-up period when the formation of aerobic granular biomass and its evolution were

studied. Along this period the OLR was maintained between 2 and 5 kg $COD_S/m^3 \cdot d$. On Stage II (days 296-330) a study of the aerobic granules stability was performed by means of the application of variable OLRs between 3 and 13 kg $COD_S/m^3 \cdot d$.

**Table 3.2.** Composition of the feeding in the different stages.

| Stage (d) | $COD_T$ (mg/L) | $COD_S$ (mg/L) | $NH_4^+$-N (mg/L) | $PO_4^{3-}$-P (mg/L) | TSS (mg/L) | VSS (mg/L) | Conductivity (mS/cm) | pH |
|---|---|---|---|---|---|---|---|---|
| IA (0-90) | 902±239 | 785±194 | 74±25 | 32±5 | 59±40 | 54±41 | 4.0±2.4 | 6.7±0.3 |
| IB (91-130) | 1076±250 | 931±189 | 112±27 | 40±7 | 122±98 | 109±87 | 4.6±1.4 | 6.7±0.6 |
| IC (131-180) | 476±96 | 462±88 | 56±11 | 19±4 | 39±11 | 30±11 | 1.8±0.3 | 6.9±0.1 |
| ID (181-295) | 870±172 | 785±183 | 90±22 | 60±10 | 63±22 | 55±20 | 2.9±0.7 | 6.9±0.2 |
| IIA (296-303) | 2538±506 | 1775±278 | 253±113 | 44±5 | 93±10 | 80±8 | 4.7±0.8 | 6.7±0.1 |
| IIB (304-315) | 958±114 | 796±85 | 96±11 | 34±6 | 90±17 | 80±13 | 2.4±0.2 | 6.6±0.1 |
| IIC (316-321) | 3262±548 | 2808±562 | 250±81 | 51±9 | 190±34 | 160±28 | 6.1±0.1 | 6.6±0.1 |
| IID (322-330) | 871±193 | 662±182 | 102±19 | 8±11 | 48±19 | 47±15 | 2.5±0.2 | 6.7±0.1 |

### 3.3.3. Inoculum

The SBR was inoculated with 500 mL of activated sludge from the biological reactor operated in the own seafood industry, characterized by a Sludge Volume Index (SVI) of 125 mL/g TSS and a solids concentration of 3.21 g VSS/L.

### 3.3.4. Analytical methods

The pH, conductivity, DO, ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS) and SVI were determined according to the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The phosphate ($PO_4^{3-}$) was determined by ion chromatography. Chemical Oxygen Demand (COD) was determined by a semi-micro method (Soto *et al.*, 1989); total COD ($COD_T$) was measured directly in the sample and the soluble COD ($COD_S$) from the sample filtered through 0.45 μm pore size filters. The protein content was determined by the Folin-Lowry method (Lowry *et al.*, 1951) and expressed in equivalent of Bovine Serum Albumin (BSA). 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). Biomass density, in terms of g VSS per litre of granules, was determined with dextran blue according to the methodology proposed by Beun *et al.* (1999).

## 3.4. RESULTS AND DISCUSSION

### 3.4.1. Granules formation and stability

During the first sixteen days of operation the settling time in the reactor was fixed at 3 minutes which implied that only the biomass with a settling velocity higher than 3.2 m/h was retained in the system. Then it was changed to 1 minute to promote a better washout of flocculent biomass with a settling velocity lower than 9.5 m/h.

After 21 days of operation the formation of the first aggregates with a filamentous surface (Figure 3.2.A) was observed. They presented an average diameter of 1.8 mm, a SVI around 125 mL/g TSS and a density of 15 g VSS/$L_{granule}$ (Stage IA; Figure 3.3). Then the diameter of these aggregates progressively increased to 5.4 mm around day 90 (Stage IB), while the SVI and the density slightly varied. The biomass concentration in the reactor was between 1 and 2 g VSS/L in Stages IA and IB, while the solids concentration of the effluent was relatively high around 0.3-0.7 g VSS/L (Figure 3.4).

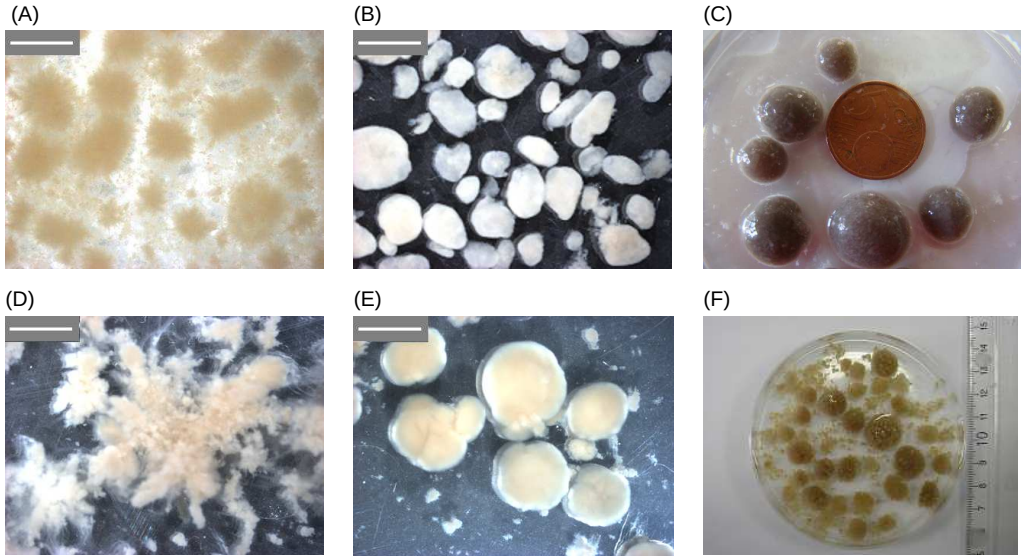


**Figure 3.2.** Images of the granular biomass in operational days: 21, Stage IA (A); 170, Stage IC (B); 219, Stage ID (C); 263, Stage ID (D); 280, Stage ID (E) and 315, Stage II (F). The size bar corresponds to 3 mm.

These aggregates were not resistant structures and from day 130 of operation (Stage IC) gradually disappeared to give rise to granular biomass. The SVI began to decrease down to 100 mL/g TSS, the density to increase up to 25 g VSS/$L_{granule}$ (Figure 3.3) and the solids to accumulate until reaching values of 8 g VSS/L (Figure 3.4). Accordingly, the concentration of solids in the effluent decreased to 0.1 g VSS/L. Aerobic granules predominated inside the reactor with a smooth surface and compact structure on Stage IC (Figure 3.2.B), that presented

an average diameter of 2.8 mm and good settling properties (SVI of 35 mL/g TSS and density of 60 g VSS/$L_{granule}$) around day 170 of operation.

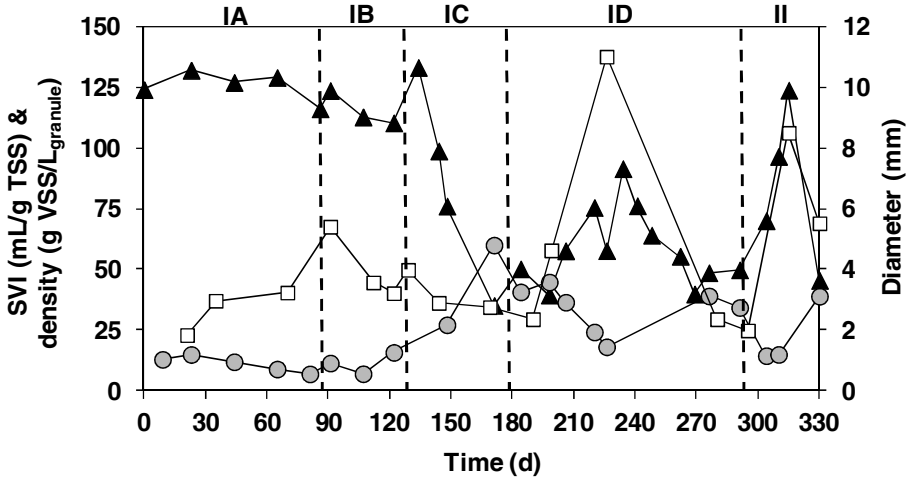


**Figure 3.3.** Evolution of the SVI (mL/g TSS) (▲), average diameter (mm) (□) and density (g VSS/$L_{granule}$) (●) of the granules along the different operational stages.

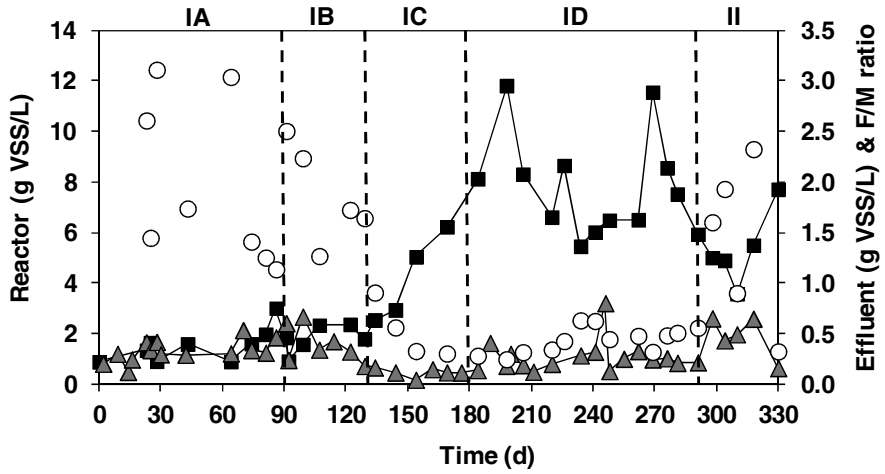


**Figure 3.4.** Concentrations of biomass (g VSS/L) inside the reactor (■) and in the effluent (▲) and F/M ratio (g CODs/g VSS·d) (○) along the different stages.

In Stage IC the physical properties of the granules were similar to those obtained by other authors working with industrial wastewater (Arrojo *et al.*, 2004; Schwarzenbeck *et al.*, 2005; Figueroa *et al.*, 2008). But from day 180 (Stage ID), coinciding with a new change of the feeding composition, the granules began to grow disproportionately (Figure 3.2.C) reaching on day 226 an average diameter of 11 mm and a few granules a maximum value of 17 mm. This increase in

size, which took place in only a few days, could be related to an increase of the residual levels of coagulant-flocculant reagents in the feeding (Guo *et al.*, 2010) due to a failure in the mixing system of the pre-treatment unit from the seafood industry. This size increment led to a worsening of the settling properties of the biomass: the SVI increased up to 91 mL/g TSS and the density diminished to 18 g VSS/$L_{granule}$. Toh *et al.* (2003) also observed that the density started to decrease when the size reached a certain limit value (4 mm of diameter) and that the biggest granules had the highest SVI values. According to this authors this evolution was caused by the fact that the packing of the big granules is less effective than that of the small size ones. At this point in the present work the big size of the granules caused a bad packing of the sludge bed and consequently after settling the biomass reached the level of the effluent port. For this reason a purge of biomass occurred involving the decrease of the VSS concentration inside the reactor observed on day 200 of operation (Figure 3.4). Furthermore this purged biomass corresponded to the smaller aggregates that were placed on the top of the sludge blanket after settling (Figure 3.5.A), which provoked that the bigger granules predominated inside the reactor (Figure 3.5.B).

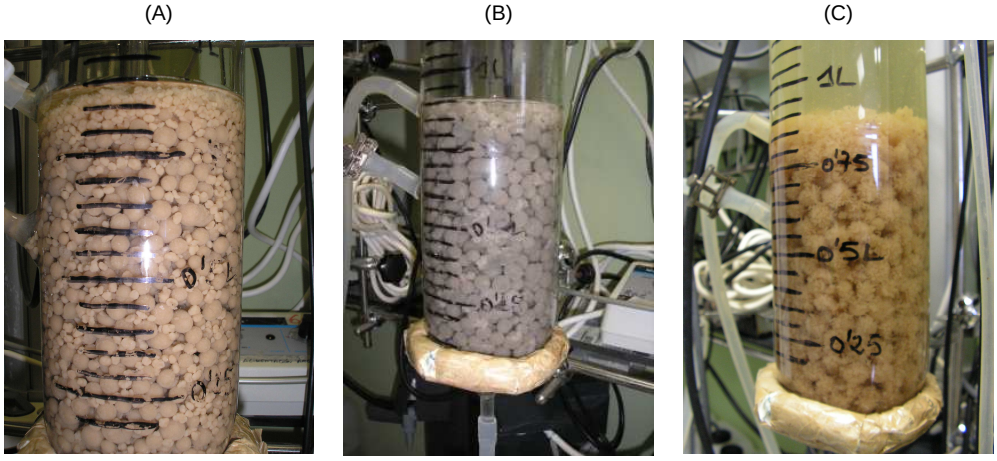


**Figure 3.5.** Photos of the bed of granules after settling inside the reactor on days 199, Stage IC (A); 226, Stage IC (B) and 304, Stage ID (C).

Previous observations about the evolution of the average diameter of the granules can be analysed in terms of volume contribution of each fraction to the total volume occupied by the biomass. The granules size distributions along different days of operation of Stage I are compared in terms of volume percentage in order to determine the sizes which contribute the most to the biomass concentration in the reactor (Figure 3.6). Most of the volume percentage of the biomass on day 21 (Stage IA) corresponded to granules with a size distribution between 2.5 and 3.0 mm. However on day 144 (Stage IC) the volume percentage was shifted to diameters between 4.5 and 5.5 mm indicating the higher contribution of the big granules to the biomass concentration in the system. The size distribution on day 199 (Stage ID) of operation indicates that two types of granules were inside the reactor, one with a distribution around 4.0 mm and other between 8.5 and 11.0 mm with a higher contribution in terms of volume percentage. The

presence of two different distributions can be appreciated in the reactor image after the sedimentation phase (Figure 3.5.A), where the smaller granules were placed in the spaces between the bigger ones and on the top of the sludge blanket. Due to the wash out of the smaller granules on the successive cycles the size distribution changed and on day 226 the granules with a size between 10.0 and 12.0 mm had the major contribution to the total biomass volume (Figure 3.6).

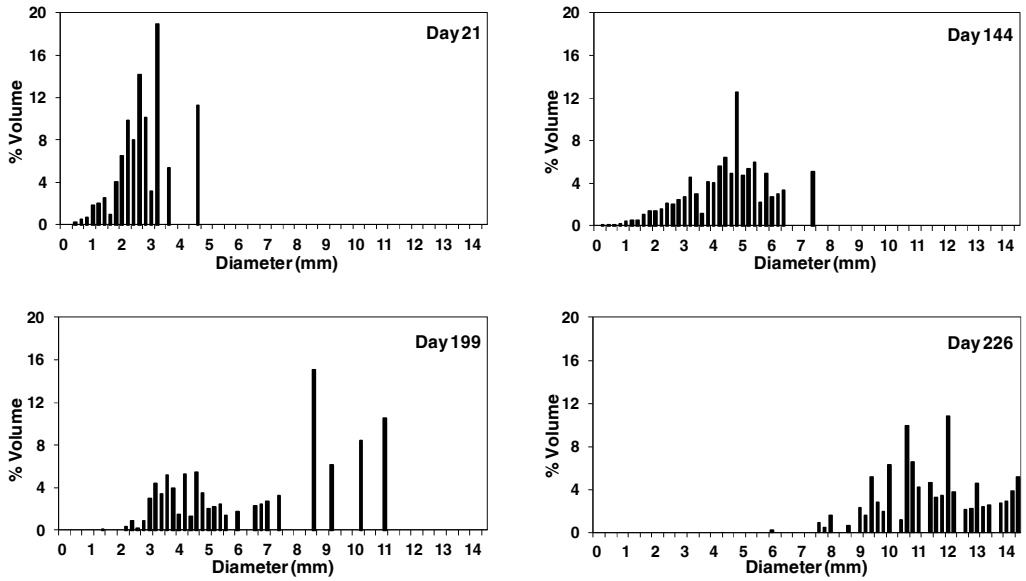


**Figure 3.6.** Volume size distribution of the aerobic granular biomass on different days of operation.

Around day 240 of operation (Stage ID) the granules started to break up into small pieces and subsequently an increase of the solids concentration in the effluent up to values around 0.8 g VSS/L was observed (Figure 3.4). Zheng *et al.* (2006) also observed the disintegration of the aerobic granules when they reached a diameter of 16 mm and the biomass was washed out with a consequent failure of the reactor operation. These authors explained that mass transfer limitations and the possible presence of anaerobic biomass in the inner part of the granules provoked this phenomenon. In this study the biomass retained in the reactor (Figure 3.2.D) served as inoculum for new aggregates formation which began on day 270 of operation, leading to the increase in the biomass concentration up to 11.6 g VSS/L. At this concentration the biomass reached again the level of the effluent port, so a new purge was performed in order to avoid the presence of high concentrations of solids in the effluent. The new granules (Figure 3.2.E) presented on day 280 an average diameter of 2.4 mm, a SVI of 49 mL/g TSS and a density of 39 g VSS/$L_{granule}$.

From day 296 till the end of the operational period a variable OLR was applied to the system (Stage II) to simulate the real conditions of the wastewater composition produced in the

industry and to determine whether the aerobic granular system was capable to maintain stable operational conditions with fluctuating loads. Between days 290 and 295, with an applied OLR around 3.5 kg $COD_S/m^3 \cdot d$, the granules had an average diameter of 2.0 mm, a SVI of 50 mL/g TSS and a density of 34 g $VSS/L_{granule}$. However the increase of the OLR up to 9 and 13 kg $COD_S/m^3 \cdot d$ on days 300 and 315, respectively, caused that the granules started to grow in size until reaching 9.0 mm, which supposed a worsening in the settling properties (SVI of 124 mL/g TSS and density of 15 g $VSS/L_{granule}$). Furthermore the aspect of the granules changed, the new biomass grew as small aggregates attached to the surface of the old granules and their surface became rough (Figure 3.2.F and Figure 3.5.C). These aggregates were not stable and again they broke up on day 320. Nevertheless at the end of the operational period, when an OLR around 2.5 kg $COD_S/m^3 \cdot d$ was applied, from the broken particles a new granulation process occurred producing granules with an average diameter of 5.5 mm, SVI of 46 mL/g TSS and density of 39 g $VSS/L_{granule}$.

So along the whole operation of the SBR with the effluent from the seafood industry the aerobic granular biomass experienced successive stages of granules formation and breakage, which are summarized in Table 3.3. The wastewater from a seafood plant used in this work presented a variable composition of the different collected batches due to the different products processed in the industry (prawn, squid, hake, etc.) and this affected the evolution of the aerobic granules physical characteristics. Despite that the first aggregates were observed on day 21 of operation (Stage IA), it was from day 130 (Stage IC), when an OLR of 2.5 kg $COD/m^3 \cdot d$ was applied, that the granulation process was achieved with a clear improvement on the settling characteristics of the biomass aggregates (Figure 3.3). Previous research works indicate that the required time to obtain the granulation and the necessary applied OLR depend on the type of substrate used to feed the reactor (Table 3.1). When synthetic media is used full granulation can be achieved after several days of operation, while the formation of aerobic granules with industrial effluents can take several months depending on the organic carbon source. Figueroa *et al.* (2008), using a similar effluent to that used in this work coming from a fish caning industry, needed 75 days to obtain mature aerobic granular biomass at OLR of 1.5 kg $COD/m^3 \cdot d$, although the effluent composition was not so variable as it is in the present work.

**Table 3.3.** Summary of the formation and breakage events of the aerobic granular biomass.

| Stage - Day of operation | Process | OLR (kg $COD_S/m^3 \cdot d$) | F/M (kg $COD_S$/kg VSS·d) | Diameter (mm) |
|---|---|---|---|---|
| IC-130 | 1st granulation | 2.5 | 0.9 | 4.0 |
| ID-246 | 1st breakage | 4.0 | 0.6 | 11.0* |
| ID-280 | 2nd granulation | 4.0 | 0.5 | 2.4 |
| IIC-320 | 2nd breakage | 13.0 | 2.3 | 9.0* |
| IID-330 | 3rd granulation | 2.5 | 0.3 | 5.5 |

*Granules average diameter before the breakage events.

Another important parameter which influences the formation of aerobic granular biomass is the Food-to-Microorganism (F/M) ratio (Li *et al.*, 2011). The F/M ratio in the SBR, before the

granulation process started, was over 1 g $COD_s$/g VSS·d (Figure 3.4). However when the aerobic granules predominated in the system and the solids concentration increased the F/M ratio decreased. The granulation process took place at a F/M ratio around 0.9 g $COD_s$/g VSS·d (Table 3.3) and it was maintained between 0.3-0.6 g $COD_s$/g VSS·d in Stages IC and ID. The adequate ratio for aerobic granulation depends on the type of wastewater used and its biodegradable fraction. In Table 3.1 it is observed that values of the F/M ratio equal or lower than 1 g $COD_s$/g VSS·d are common to obtain aerobic granular biomass. However with acetate Adav *et al.* (2010) could achieve the granulation at a high value of the F/M ratio (2.4 g $COD_s$/g VSS·d), due to the use of a easy biodegradable source of organic carbon. On the other hand Figueroa *et al.* (2011) treating swine slurry needed a lower value of the F/M ratio (0.5 g $COD_s$/g VSS·d) to obtain aerobic granules probably due to the low biodegradability of this type of effluent.

With all this previous information it is possible to analyse the subsequent formation and breakage events. The first instability and breakage event of the aerobic granular biomass was around day 246 (Stage ID) due to the presence of high residual levels of coagulant-flocculant reagents that provoked a high increase on the granules average diameter in few days, despite that the OLR was not so high and the F/M ratio was below 1 g $COD_s$/g VSS·d (Table 3.3). Tay *et al.* (2006) explained that a fast growth of the aerobic granules (> 5 mm) with filamentous surface takes place when a rich medium in nutrients is present. Furthermore some works showed that big aerobic granules can be obtained when the feeding is supplemented with cations like $Ca^{+2}$ (Jiang *et al.*, 2003) and $Mg^{+2}$ (Li *et al.*, 2009) which present similar properties to the coagulant reagents.

The second instability and breakage event was due to an increase of the F/M ratio to 2.3 g $COD_s$/g VSS·d (Table 3.3) provoked by the application of high and variable OLRs (Stage II) and the reduction in the solids concentration. Before this breakage the physical properties of the aggregates worsened: the average diameter and the SVI increased while the density decreased (Figure 3.3). This coincides with the results obtained by Li *et al.* (2011) who tested the effect of the F/M ratio, with values between 0.3 and 1.1 g COD/g SS·d, on glucose fed aerobic granules. These authors observed that a high value of this ratio provoked the faster formation of larger granules with a worse sludge compressibility compared with small size ones.

The breakage of the granules provoked an increase of the solids concentration in the effluent, which can suppose a problem of effluent quality during the application of aerobic granular systems at full scale. To avoid this problem a selective purge can be applied when the granules reach certain size and before their disintegration. In this sense, the use of a filter after the withdrawal could be also a suitable option to diminish the presence of solids in the effluent (Arrojo *et al.*, 2004).

### 3.4.2. Organic matter and nitrogen removal

The reactor was operated during 330 days treating different OLRs. During the first 90 days of operation (Stage IA) the fed OLR was around 3.0 kg $COD_S/m^3$·d and the obtained removal

efficiency was of 85% for $COD_S$ (Figure 3.7). Then the applied OLR increased up to 4.5-5.0 kg $COD_S/m^3 \cdot d$ due to a change of the feeding concentration (Stage IB) and the efficiency of organic matter removal decreased to values of 50% during the first days of this period, maybe due to an increase in the slowly or non biodegradable fraction of the organic matter. On day 130 with a new batch of industrial wastewater the OLR applied was of 2.5 kg $COD_S/m^3 \cdot d$ (Stage IC) which supposed a reduction in the F/M ratio to values lower than 1 g $COD_S$/g VSS·d, the disappearance of flocculent biomass and the prevalence of granular biomass. Then the OLR in the influent was gradually augmented from 2.5 kg $COD_S/m^3 \cdot d$ (day 180) to 4.4 kg $COD_S/m^3 \cdot d$ (day 270), with a removal efficiency of 90% for $COD_S$ (Stage ID). Results obtained in the present study were in accordance with Figueroa *et al.* (2008) who obtained removal efficiencies of 90-95% for $COD_S$ treating a similar industrial effluent but at lower applied OLR (1.6 kg $COD/m^3 \cdot d$).

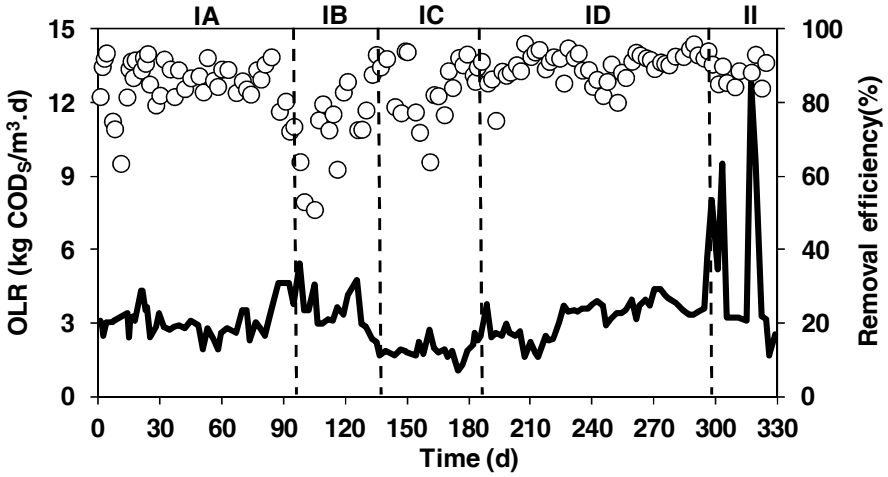


**Figure 3.7.** Profile of OLR (-) and percentage of $COD_S$ removal (○ ) during the different stages of operation.

In order to determinate if the aerobic granular system was capable of maintaining stable operational conditions with fluctuating loads (Stage II), the OLR was suddenly increased from 3 to 9 kg $COD_S/m^3 \cdot d$ on day 296 (Stage IIA) and restored to the previous organic load on day 303 (Stage IIB). Again, the following week (Stage IIC), the organic load was increased up to 13 kg $COD_S/m^3 \cdot d$. During this stability test, despite the decrease in the solids concentration and the increase up to 1 g $COD_S$/g VSS·d of the F/M ratio (Figure 3.4), the removal of $COD_S$ was kept similar to the rest of the operational period with values between 85% and 90%. Thereby the system was capable to maintain the removal efficiency of organic matter, even during the sudden applied OLRs, indicating a rapid response of the granular biomass to cope with the variable loads during the feast phase.

The NLR fed to the SBR varied between 0.2-0.6 kg $NH_4^+$-$N/m^3 \cdot d$ on Stage I and 0.3-1.5 kg $NH_4^+$-$N/m^3 \cdot d$ on Stage II (Figure 3.8). The removal efficiencies of Total Nitrogen (TN) and ammonia were also variable, with the maximum values of 30 and 65%, respectively.

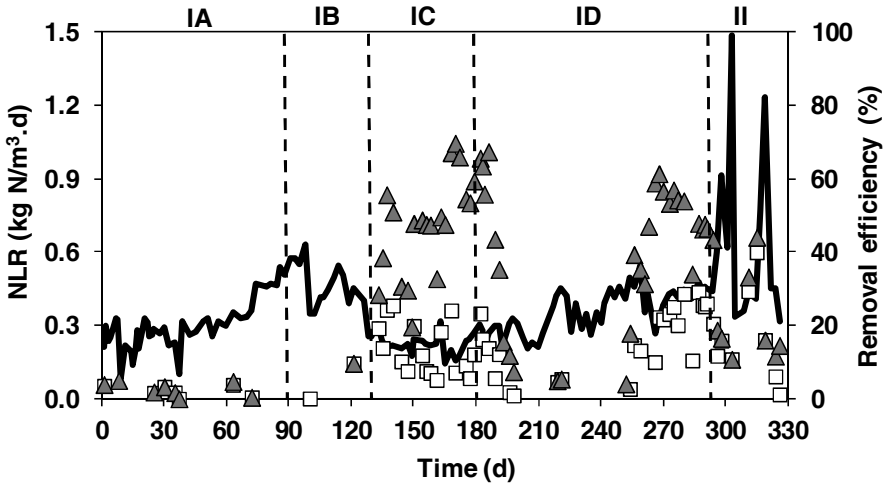


**Figure 3.8.** Profile of applied NLR (-), removal percentages of TN (□ ) and $NH_4^+$ (▲).

In order to know the limiting factor in the ammonia removal process along the operation its percentage was represented as a function of the Solids Retention Time (SRT), the Free Ammonia (FA) concentration and the applied Superficial Nitrogen Loading Rate (SNLR) (Figure 3.9). From these relations it was found that the ammonia removed was limited by SRT values lower than 4 days, FA concentrations higher than 4 mg $NH_3$-N/L and applied SNLR higher than $10^{-3}$ kg $NH_4^+$-N /$m^2$·d. The minimum SRT necessary for the nitrification at the operating temperature (22 ± 2 ºC) is, according to the literature, around 3-4 days (Salem *et al.*, 2003). Respect to the FA concentration Yang *et al.* (2004) found that the nitrification was completely inhibited at a concentration greater than 10 mg $NH_3$-N/L and that the specific oxygen utilization rate of nitrifying bacteria was reduced by a factor 5 and 2.5 as the FA concentration increased from 2.5 to 39.6 mg $NH_3$-N/L. De Kreuk *et al.* (2007) observed that the low specific surface availability became limiting for oxygen transport and thus for the ammonia oxidation process.

To identify which variable limited the ammonia removal for each stage their values are presented in Table 3.4. On Stage IA the ammonia oxidation did not occur and even the concentration in the effluent was higher than in the influent in some days due to the hydrolysis of the proteins present in the fed wastewater, as could be checked measuring their concentration in the influent (between 100 and 300 mg/L of BSA) and in the effluent of the reactor (between 10 and 50 mg/L of BSA). The low SRT and the slightly high FA concentration were the responsible parameters for the ammonia removal absence. On Stage IB the SRT, FA concentration and applied SNLR were unfavourable. On Stage IC, coinciding with the granulation process, the ammonia removal percentage was around 60%. The achieved high biomass concentration inside the reactor with the aerobic granular biomass (Figure 3.4) supposed an increase in the SRT from 2 days (at the end of Stage IB) to 13 days (at the end of Stage IC), which favoured the retention of microorganisms with relatively slow growth rates, such as nitrifying bacteria, and promote the ammonia removal. Besides during this period the values of FA concentration and

applied SNLR were low which also promoted the nitrification process. However the denitrification process was no favoured which resulted in the accumulation of $NO_x^-$ compounds, therefore the TN removal was only around 20-30% on Stage IC (Figure 3.8). At the beginning of Stage ID, as the aerobic granules increased in size and its specific surface decreased the ammonia removal was worsened. However the ammonia removal process took place again from day 250 (Stage ID), due to the breaking up of the previous granules which increased the surface available to oxygen transfer.

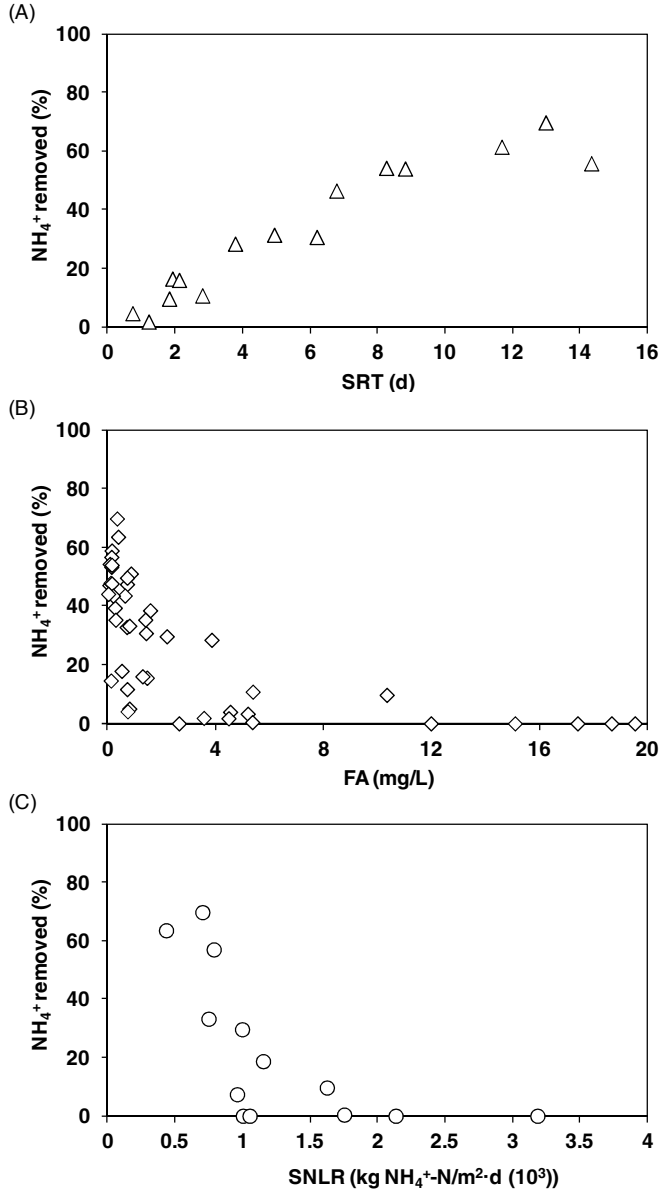


**Figure 3.9.** Relation between $NH_4^+$ removed and SRT (△), FA (◇) and SNLR (○).

On Stage II (stability test) the ammonia oxidation was around 15% when the applied NLR was high (1.5 kg $NH_4^+$-N/$m^3$·d) and around 40% when the NLR was low (0.3 kg $NH_4^+$-N/$m^3$·d), which indicated that the system had not capacity to treat variable NLRs. The decrease in the solids concentration inside the reactor at the beginning of the Stage II that implied a reduction in the SRT and the increase in the applied SNLR provoked the decrease in the ammonia removal efficiency.

**Table 3.4.** Parameters influencing the nitrification process.

| Stage | SRT (d) | FA (mg/L) | kg $NH_4^+$-N/$m^2$·d ($10^3$) |
|---|---|---|---|
| IA | 1.1 ± 0.4 | 5.2 ± 2.7 | 1.4 ± 0.5 |
| IB | 1.4 ± 0.7 | 15.0 ± 5.1 | 2.3 ± 0.8 |
| IC | 3.8 – 13.0 | 1.1 ± 0.6 | 0.8 ± 0.2 |
| ID | 4.8 – 16.0 | 0.4 – 9.2 | 0.8 ± 0.3 |
| II | 2.2 ± 0.4 | 0.2 – 5.4 | 0.3 – 1.5 |

### 3.4.3. Compounds profile along an operational cycle

The profiles of different compounds concentrations were also analysed during some operational cycles to obtain a better understanding of the processes involved in both organic matter and nitrogen removal. As an example the profiles on the operational days 72 (Stage IA) and 290 (Stage ID) are shown in Figure 3.10.

When the process of granulation did not occur (day 72) the $COD_S$ readily biodegradable was removed in 60 minutes (long feast period). During this period of operation, the nitrification and denitrification processes did not occur, the ammonia concentration only decreased from 95 to 89 mg $NH_4^+$-N/L due to the biomass growth and nitrite and nitrate did not appear. The DO concentration was around 6.5 mg $O_2$/L during the feast period and then increased to 7.5-8.0 mg $O_2$/L until the end of the cycle.

On day 290 the biomass was in the form of aerobic granules and the profiles of the soluble compounds along the cycle were different. The $COD_S$ readily biodegradable only took 20 minutes to be eliminated, which implies a short feast period compared to the flocculent biomass. The nitrification process occurred and ammonia was oxidised to nitrite, and nitrite to nitrate, during the aerobic period immediately after the disappearance of the biodegradable organic matter from the liquid phase. Part of the nitrite and nitrate accumulated at the end of the cycle were consumed via denitrification at the beginning of the cycle. The DO concentration was in the first minutes (feast period) around 6.3 mg $O_2$/L, and during the rest of the cycle (famine period) near the saturation value (8-9 mg $O_2$/L).

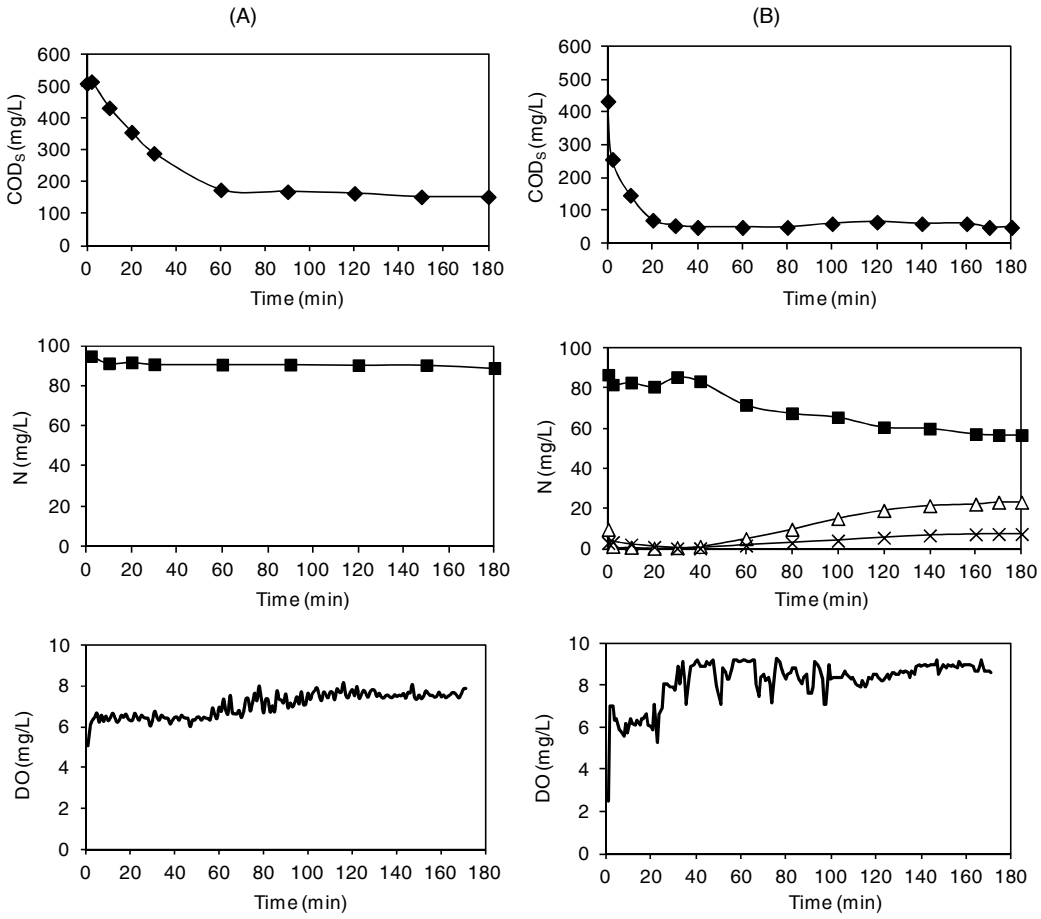


**Figure 3.10.** Concentration profiles during an operational cycle of the SBR on day 72 (A) and 290 (B). Concentrations of $COD_S$ (◆), $NH_4^+$-N (■), $NO_2^-$-N (△), $NO_3^-$-N (×) and DO (-).

### 3.4.4. Biomass yield

Aerobic granular systems are characterized by a lower biomass production compared to CAS systems and this fact is related to the higher sludge age achieved with aerobic granular biomass (Campos *et al.*, 2009b). In this chapter the yield of the microorganisms (Y) expressed in terms of gram of biomass produced per gram of COD consumed (g VSS/g COD) was calculated for different periods of operation and represented as a function of the SRT (Figure 3.11).

When the flocculent biomass was dominant inside the reactor (days 0-130) the estimated yield was between 0.45 and 0.65 g VSS/g $COD_{removed}$, which corresponded with SRT values around 1-2 days. These results are comparable with those from CAS systems with typical biomass yields of 0.4-0.6 g VSS/g $COD_{removed}$ (Droste, 1996). Since granulation process

occurred and the SRT increased up to 4 days the yield decreased down to 0.30 g VSS/g $COD_{removed}$. Therefore the biomass yield corresponding to aerobic granules was 54% lower than that obtained when the SBR contained flocculent biomass. Other authors obtained similar values of biomass yield (between 0.20 and 0.33 g VSS/g $COD_{removed}$) for aerobic granular systems (de Kreuk *et al.*, 2005; Liu *et al.*, 2005; Figueroa *et al.*, 2011).

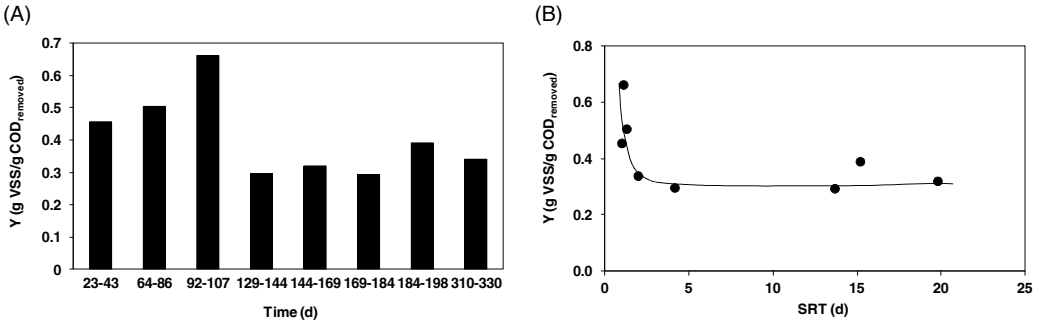


**Figure 3.11.** Evolution of microorganisms yield (Y) with the SBR operation (A) and as a function of the SRT (B).

## 3.5. CONCLUSIONS

The formation of aerobic granular biomass was achieved in a SBR treating an industrial wastewater coming from a seafood industry with a previous physical-chemical treatment. The granulation process was considered completed on day 130 of operation at an applied OLR and a F/M ratio of 2.5 kg $COD_S/m^3$·d and 0.9 g $COD_s$/g VSS·d, respectively. The time necessary to obtain aerobic granular biomass was associated with the complex nature of the industrial wastewater.

The obtained aerobic granular biomass presented good settling characteristics (SVI of 35 mL/g TSS and density of 60 g VSS/$L_{granule}$) but was submitted to successive formation and breakage events. The first breakage event was presumably due to the presence of high residual levels of coagulant-flocculant reagents in the treated wastewater that provoked a high increase on the granules average diameter in few days. In this case a further study of the effect of these compounds on aerobic granular biomass is needed. The second breakage event was presumably due to an increase of the F/M ratio to values of 2.3 g $COD_s$/g VSS·d provoked by the application of high and variable OLRs (Stage II).

The reactor was able to treat OLRs between 2 and 13 kg $COD_S/m^3$·d with a removal efficiency of around 90%. The ammonia removal was not constant along the whole operational period and was affected by the SRT value, FA concentration and applied SNLR. The maximum percentage of ammonia and TN removal reached were 65% and 30%, respectively.

The application of high and variable loads (Stage II) did not affect the organic matter removal, but the physical properties of the biomass and the nitrogen removal worsened.

During the operation of the SBR with stable aerobic granular biomass and SRT values higher than 4 days the obtained biomass growth yield was 54% smaller than that obtained during the operation of the SBR with flocculent biomass and SRT around 1 day.

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# Chapter 4:

# Effect of coagulant-flocculant reagents on aerobic granular biomass [1]


## Summary

Technologies based on aerobic granular biomass have been found to be an alternative for the industrial wastewater treatment due to their advantages in comparison with the Conventional Activated Sludge (CAS) systems. However the operation of the former can be influenced by the presence of residual amounts of compounds such as coagulant-flocculant reagents, frequently used as pre-treatment before the biological process, which could affect the aerobic granular biomass formation and its physical properties. In previous Chapter 3 the application of an industrial effluent pre-treated with these compounds as a feeding to a Sequencing Batch Reactor (SBR) made feasible the formation of aerobic granular biomass. In that case the stability of the formed aggregates seemed to depend on the presence of these compounds. Although the complex nature of industrial effluents, like those tested from a seafood processing plant, makes it impossible to clearly confirm this hypothesis, since another compounds present in the wastewater could contribute to the observed negative effect on the stability of aerobic granular biomass. For this reason in this chapter the effects of residual amounts of coagulant-flocculant reagents present in the feeding media were tested in terms of organic matter and nitrogen removal efficiencies and granular biomass physical stability.

Two aerobic granular sludge reactors were operated, one fed with a synthetic medium containing residual amounts of coagulant and flocculant reagents (CF) and another as control fed with a medium without these compounds addition. The results showed that the presence of coagulant-flocculant reagents led to a worse biomass retention capacity allowed the accumulation of a lower VSS concentration compared to those values obtained in the control reactor (4.5 vs. 7.9 g VSS/L) and the biomass presented a higher SVI (70 vs. 40 mL/g TSS) and average diameter (5.0 vs. 2.3 mm). The visual aspect of the aerobic aggregates was also different with a fluffy and filamentous surface when coagulant-flocculant reagents were present which could be related to the lower values of the famine/feast ratio observed in comparison with those from the control reactor. These reagents also caused a decrease in the maximum oxygen consumption rate but the removal efficiencies of organic matter (90%) and nitrogen (60%) were not affected.


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[1] **Val del Río, A.**, Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J.L. and Méndez, R. (2012). Effect of coagulant-clocculant reagents on aerobic granular biomass. *Journal of Chemical Technology & Biotechnology*, 87(7), 908-913.

## 4.1. INTRODUCTION

Technologies based on aerobic granular sludge appear as a promising option for their application to the wastewater treatment (de Bruin *et al.*, 2004; Liu and Tay, 2004). Advantages in comparison to the CAS systems rely on their capacity to treat higher loads, to achieve better removal efficiencies, to carry out simultaneous elimination of organic matter, nitrogen and phosphorus (de Kreuk *et al.*, 2005) and to produce biomass with better settleability properties and lower sludge production. Despite all the advantages of the aerobic granular biomass systems the development of this technology is still recent and the research at laboratory scale was focused on the establishment of the optimal operational parameters such as feeding strategy (feast/famine regime), reactor design (large high/diameter ratio), low settling time, aeration intensity (high hydrodynamic shear force), control of the Dissolved Oxygen (DO) concentration, etc. The study of different types of substrates to obtain aerobic granular biomass is also important to determine the usefulness of this technology and its application to the different types of effluents. Many works have been performed with synthetic (Beun *et al.*, 1999; Tay *et al.*, 2002; de Kreuk *et al.*, 2005) and industrial wastewater (Arrojo *et al.*, 2004; Schwarzenbeck *et al.*, 2004; Cassidy and Belia, 2005; Inizan *et al.*, 2005; Figueroa *et al.*, 2008). The effect of different types of toxic compounds, like phenol and pyridine as well as the presence of heavy metals and dyes have also been tested (Adav *et al.*, 2008; Moussavi *et al.*, 2010). However the effects of the presence of coagulant-flocculant reagents, commonly used in the Wastewater Treatment Plants (WWTPs) as pre-treatment for solids separation before the biological one, have not been studied so far.

The coagulants are normally cationic salts designed to neutralize the repulsive electrical charges (typically negative) surrounding particles and allowing the formation of flocks, while the flocculants facilitate the agglomeration or aggregation of these coagulated particles to form larger flocks that could be easily separated from the wastewater. A residual amount of these reagents remains in the supernatant and goes to the biological treatment process, affecting its performance. In some cases the effects are positive: to hinder the formation of biological foam and the proliferation of filamentous microorganisms (Lansky *et al.*, 2005), to improve the sludge settling properties (Lees *et al.*, 2001) and to protect membranes from fouling (Pendashteh *et al.*, 2011), but they can also have negative effects on the biomass activity (Lees *et al.*, 2001; Dapena-Mora *et al.*, 2007).

Although there are not specific studies about the effect of these reagents on the formation and properties of the aerobic granular biomass, some studies with cations similar to those used as coagulants, such as $Ca^{+2}$ (Jiang *et al.*, 2003) and $Mg^{+2}$ (Li *et al.*, 2009), show that their presence favour the formation of aerobic granular biomass due to the fact that they act as a bridge to bind negatively charged groups present on bacterial surface and/or extracellular polysaccharides molecules to adhere individual bacteria to each other (Campos *et al.*, 2009). So the residual levels of coagulant-flocculant reagents could initially promote the aggregation of the biomass, but the long term effect is still unknown.

## 4.2. OBJECTIVE

The objective of this work was to study the effect of residual concentrations of coagulant-flocculant reagents on an aerobic granular system. Special attention was paid to the formation and the evolution of the biomass physical properties as well as to the removal efficiencies of organic matter and nitrogen.

## 4.3. MATERIALS AND METHODS

### 4.3.1. Experimental set-up and operational conditions

Two sequencing batch reactors (SBRs) were operated with the same experimental set-up than the SBR described in Chapter 3.

The feeding of both reactors was a synthetic medium with the following composition: NaAc·3 $H_2O$ 1.7 g/L, $NH_4Cl$ 0.3 g/L, $K_2HPO_4$ 0.12 g/L, $MgSO_4$ 0.04 g/L, $CaCl_2$·2 $H_2O$ 0.07 g/L, KCl 0.02 g/L and 1 mL/L of a trace solution according to Smolders *et al.* (1995). One reactor was used as control and the other one (CF) was supplemented with coagulant (2.5 mg/L of Polychloride of aluminium) and flocculant (1.5 mg/L of commercial polyelectrolyte Chemifloc®) according to the range of residual concentrations commonly found in the primary settled wastewater (Lees *et al.*, 2001).

The reactors were operated along 135 days maintaining the same conditions (feeding and operation) to ensure a sufficient operational period to study the possible differences of both systems performance.

### 4.3.2. Inoculum

The two SBRs were inoculated with 0.5 L of an activated sludge from an urban WWTP with a SVI around 200 mL/g TSS and a solids concentration of 5 g VSS/L. The sludge presented a fluffy, irregular and loose morphology as it was observed under the microscope.

### 4.3.3. Analytical methods

Concentrations of ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$), Dissolved Oxygen (DO), Volatile Suspended Solids (VSS), Total Suspended Solids (TSS) and Sludge Volume Index (SVI) were determined according to the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The soluble Chemical Oxygen Demand ($COD_S$) 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) and by Scanning Electron Microscopy (SEM) (Digital SEM 440, Leica). Biomass density ($\rho_b$), in terms of g VSS per litre of granules, was determined with dextran blue following the methodology proposed by Beun *et al.* (1999).

The FISH technique was used to identify filamentous and nitrifying bacteria. This analysis was performed with a set of fluorescent labelled 16S rRNA-targeted DNA probes: $EUB338_{mix}$ to

determine the bacteria domain (with sequences GCT GCC TCC CGT AGG AGT, GCA GCC ACC CGT AGG TGT and GCT GCC ACC CGT AGG TGT and a formamide concentration of 35%) and NEU653 to determine the Ammonium Oxidizing Bacteria (AOB) (with a sequence CCT CTC TGC ACT CTA TTC CAT CCC CCT CTG CCG and a formamide concentration of 40%).

### 4.3.4. Calculations

***Specific Oxygen Uptake Rate (SOUR)***

The maximum specific oxygen consumption rate ($r_{max}$) as g $O_2$/g VSS·d was calculated from the Specific Oxygen Uptake Rate (SOUR) value considering that under the operational conditions the reaction obeys a zero-order rate limited by internal diffusion [4.1] (González Velasco *et al.*, 1999):

$$SOUR=\left[1-\left[\frac{1}{2}+sen\left[\frac{1}{3}arctg\left(\frac{3\cdot\left(\frac{R_p}{3\cdot\sqrt{2}}\cdot\sqrt{\frac{r_{max}\cdot\rho_b}{D_{O2}\cdot C_{O2}}}\right)^2}{2\cdot\sqrt{3\cdot\left(\frac{R_p}{3\cdot\sqrt{2}}\cdot\sqrt{\frac{r_{max}\cdot\rho_b}{D_{O2}\cdot C_{O2}}}\right)^2-1}}\right)\right]\right]^3\cdot r_{max} \qquad [4.1]$$

Where $R_p$ is the particle radius (dm); $D_{O2}$ is the diffusion coefficient of oxygen ($dm^2$/d); $\rho_b$ is the biomass density (g VSS/$L_{granule}$) and $C_{O2}$ is the oxygen concentration in the bulk liquid (g $O_2$/L).

The SOUR (g $O_2$/g VSS·d) was calculated using the equation [4.2] along the feast phase considering only the oxidation of organic matter, since nitrification did not take place during the feast phase:

$$SOUR=\frac{k_La\cdot(C^*_{O_2}-C_{O_2})}{X} \qquad [4.2]$$

Where $k_La$ is the mass transfer coefficient ($d^{-1}$); $C^*_{O2}$ is the oxygen saturation concentration for the operational conditions of the system (g $O_2$/L); $C_{O2}$ the oxygen concentration level during the feast period (g $O_2$/L) and X is the biomass concentration inside the reactor (g VSS/L).

***Oxygen Transfer coefficient ($k_La$)***

The oxygen gas-liquid transfer coefficient ($k_La$) was estimated by means of a dynamic method (described in Chapter 2), registering the increments of DO concentrations in the SBR after the reestablishment of the aeration (without biomass in the reactor).

## 4.4. RESULTS AND DISCUSSION

### 4.4.1. Formation and characteristics of aerobic granular biomass

During the first 8 days of operation, after reactor inoculation, the settling time was maintained in both reactors at 2 minutes to avoid the excessive solids wash out, which supposed that only the particles with a settling velocity higher than 5.0 m/h were retained inside the reactors. Then the settling time was changed to 1 minute to promote a complete wash out of flocculent biomass with a settling velocity lower than 9.5 m/h. The formation of the first aggregates could be appreciated in both systems after 10 days of operation (Figure 4.1.A). These aggregates had a different evolution in the two reactors as could be observed comparing their aspect in Figure 4.1.B and Figure 4.1.C.

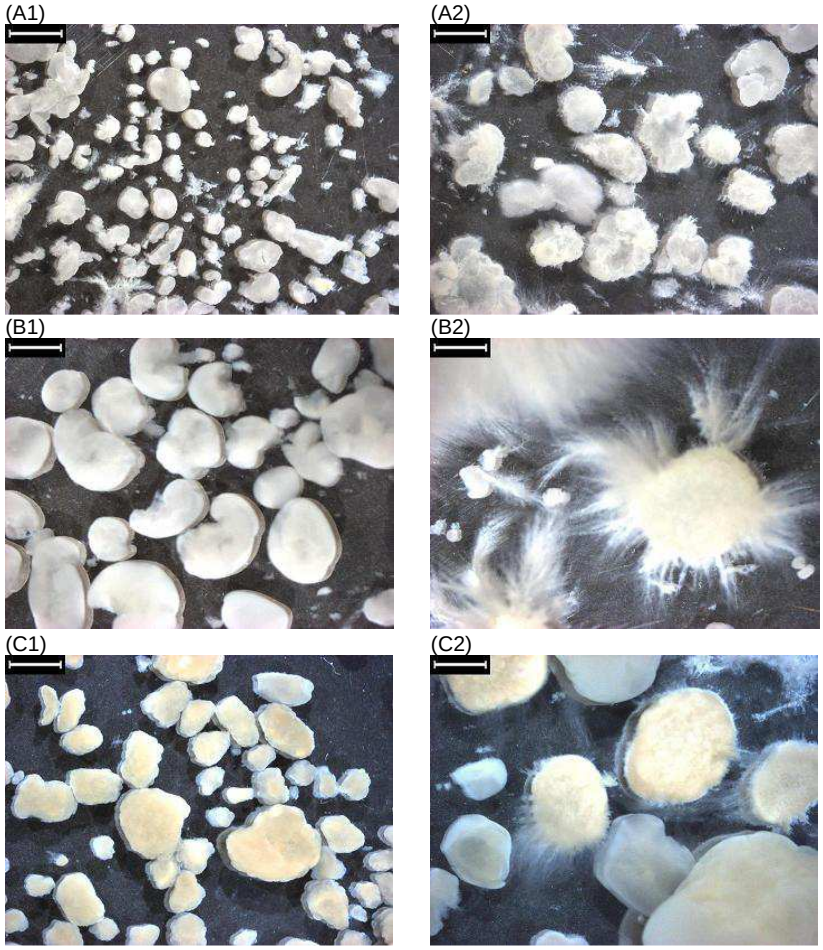


**Figure 4.1.** Images of the biomass in the control (1) and the CF (2) reactors: (A) day 10, (B) day 44 and (C) day 135. The size bar represents 2 mm.

Initially the SVI of the biomass in the control reactor decreased from 192 to around 40 mL/g TSS (day 63) but on day 70 the SVI value increased up to 130 mL/g TSS due to clogging of the air diffuser (Figure 4.2). Once it was cleaned the SVI started to gradually decrease down to 40 mL/g TSS. In the CF reactor the SVI values ranged between 120 and 70 mL/g TSS being at the end of the operation higher than those of the control reactor.

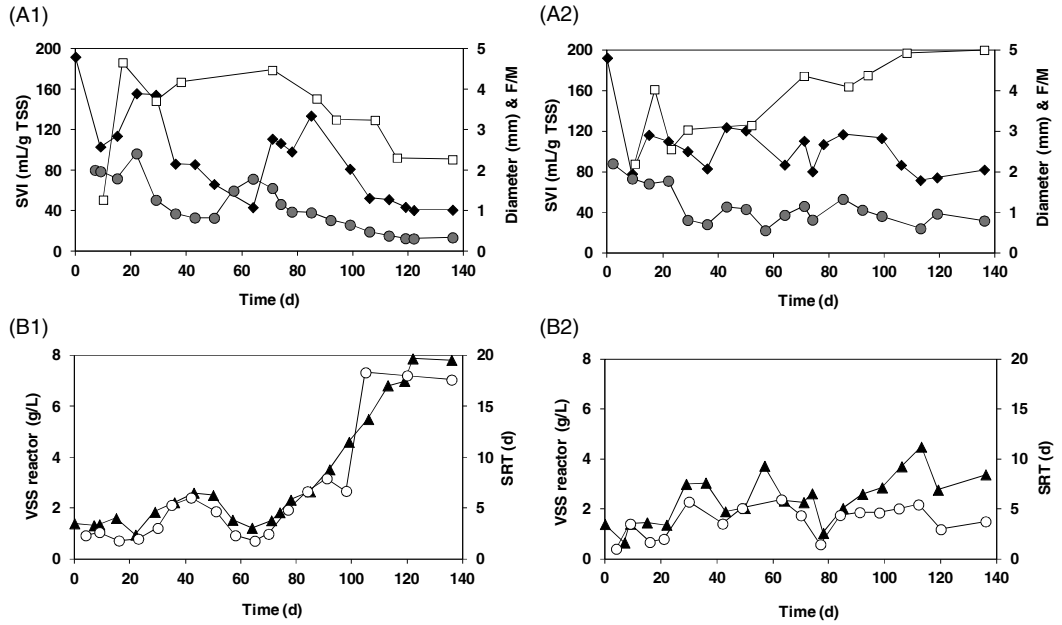


**Figure 4.2.** Biomass characteristics in the control (1) and the CF (2) reactors: (◆) SVI; (□) granules average diameter; (●) F/M ratio; (▲) VSS concentration in the reactor and (○) SRT.

The evolution of the average particle size of the granules was different in both reactors (Figure 4.2). After the first 20 days of operation, in the control reactor the granules had an average diameter of 4.7 mm which remained almost constant until day 70. Then, when the normal aeration was re-established, it gradually decreased down to 2.3 mm (day 135). In the CF reactor the particles average diameter progressively increased from 2.6 mm (day 23) to 5.0 mm (day 135). In this case the air diffuser was also cleaned to have the same operational conditions than those on the control reactor. This increase in the size of the aggregates could be due to the presence of a residual amounts of cations from the coagulant reagent, since previous works show that the addition of cations to obtain aerobic granular biomass produces granules with bigger diameter (Li *et al.*, 2009). Guo *et al.* (2010) also observed an increase in the particle size of the sludge flocs in a membrane bioreactor with the addition of three different flocculants.

At the end of the operational period the granules aspect was very different in both systems (Figure 4.1.C and Figure 4.3). Granules from the control reactor were smaller and with smoother surfaces than those from CF reactor. The different physical properties observed could attribute to the abundance of filamentous bacteria detected in the granules of the CF reactor (Figure 4.4).

A deeper analysis of the microbial community of the CF reactor (Figueroa, 2011) revealed that these filamentous bacteria corresponded to *Chloroflexi* and *Sphaerotilus natans* species. Furthermore the latter was already identified in the inoculum of both reactors. Therefore the microbial populations developed depended not only on the inoculum but also on the addition of reagents like coagulant and flocculant ones. These reagents favour the formation of aggregates which could promote the retention of the filamentous bacteria present in the inoculum and the posterior operational conditions led to their development.

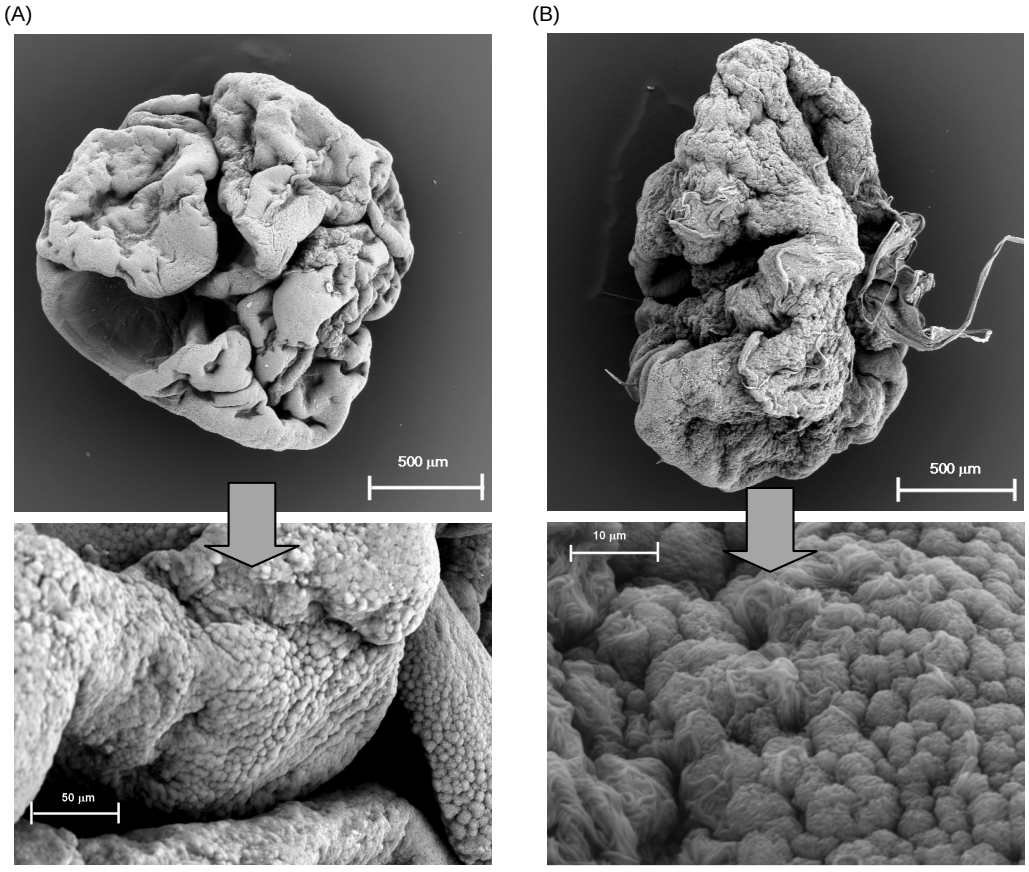


**Figure 4.3.** SEM images of an entire granule and a surface detail for the control (A) and the CF (B) reactors.

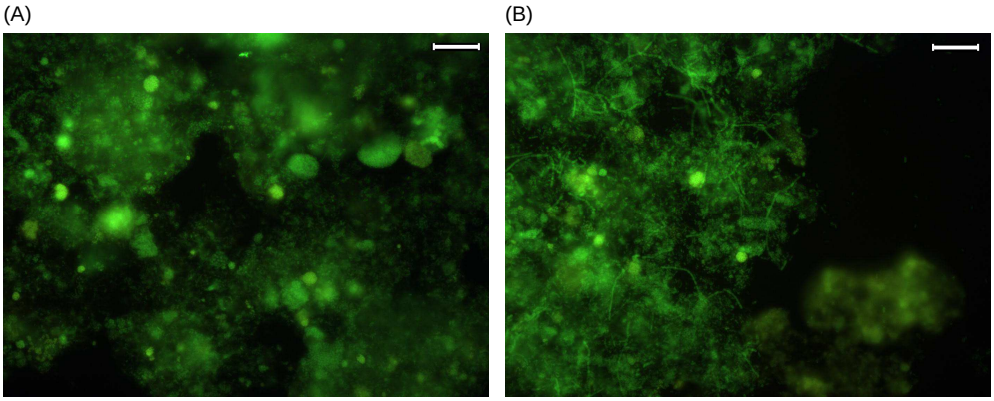


**Figure 4.4.** FISH images of EUB338$_{mix}$ (FITC) probe from (A) control and (B) CF reactors on day 135 of operation. The size bar corresponds to 25 μm.

In Figure 4.5 the granules size distribution corresponding to both reactors is presented and compared for two different days of operation. On day 87 the control and the CF reactor presented a similar volume size distribution, however at the end of the operational period (day 136) the distribution for the CF reactor was more disperse with a higher contribution of the bigger aggregates (18% of the total biomass volume was occupied by the granules with a size of 9.4 mm), while in the control reactor granules with a diameter around 2 mm represented the 20% of the volume.

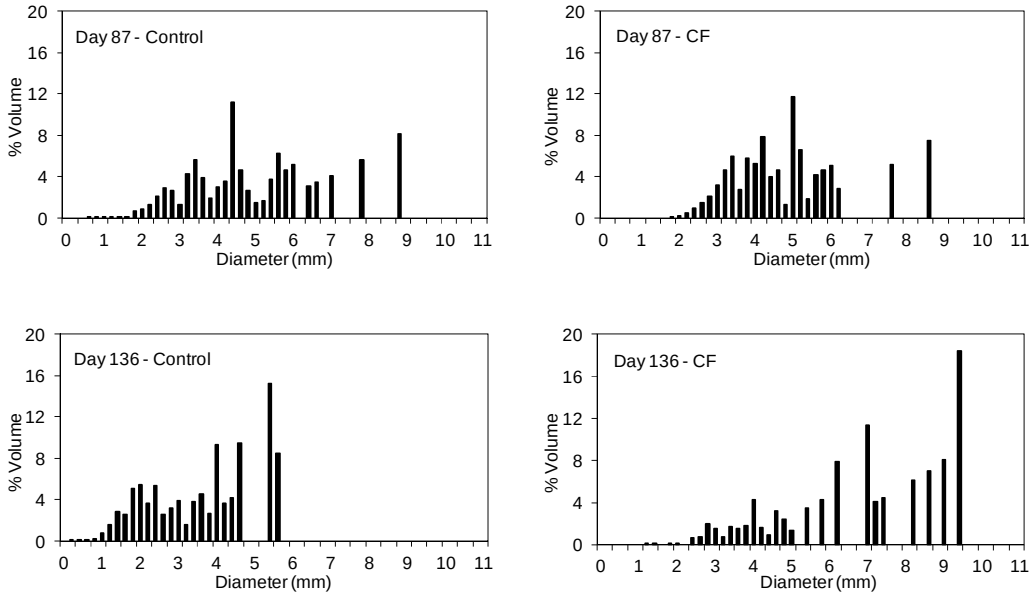


**Figure 4.5.** Volume size distribution of aerobic granular biomass on different days of operation for the two SBR reactors.

The biomass concentration inside both reactors was quite similar until day 80 of operation with values between 0.9-2.6 g VSS/L for control and 0.7-3.7 g VSS/L for CF (Figure 4.2.B). Then in the control reactor the biomass concentration started to increase up to values of 7.9 g VSS/L at the end of the operational period, while in the CF reactor the biomass concentration reached a maximum value of 4.5 g VSS/L and then it decreased to a value around 3 g VSS/L at the end of the operational period. During the first 80 days the average solids concentration in the effluent was of 0.14 g VSS/L in both reactors and the Solid Retention Time (SRT) ranged from 2 to 6 days (Figure 4.2.B). Nevertheless, from day 80 the average solids concentration in the effluent of the control reactor decreased down to 0.11 g VSS/L while in the CF reactor it increased up to 0.18 g VSS/L. The better biomass retention in the control reactor compared to the CF reactor would explain the different values of biomass concentration obtained at the end of the operational period. In both systems the biomass yield coefficient (Y) was around 0.28 g VSS/g $COD_{removed}$ for SRT values around 5 days. In the case of the control reactor, values of 0.24 g VSS/g $COD_{removed}$ were observed for SRT values around 18 days (days 100-140 of operation).

In order to determine the effect of the amount of substrate added the Food-to-Microorganism (F/M) ratio was calculated. In the case of the control reactor this parameter remained in values over or around 1 g $COD_s$/g VSS·d until day 80 (Figure 4.2.A). From this day on, due to the biomass accumulation inside the reactor, the F/M ratio progressively decreased to 0.3 g $COD_s$/g VSS·d at the end of the operational period. This operational period with values of the F/M ratio lower than 1 g $COD_s$/g VSS·d corresponded to the existence of aerobic granules with the better physical characteristics (decrease in the SVI and average diameter). However on the CF reactor the F/M ratio was around 1 g $COD_s$/g VSS·d from day 20 until the end of the operational period (Figure 4.2.A). These results are in accordance with those of Chapter 3 obtained with the industrial wastewater from a seafood industry. Therefore it was observed that for a stable granulation process the F/M ratio must be lower than 1 g COD/g VSS·d.

The main physical characteristics of the aerobic granules at the end of the experiment are summarized in Table 4.1, which evidences that the granules from the control reactor presented better physical properties than those from CF reactor, so the presence of coagulant-flocculant reagents caused a detrimental in the physical properties of aerobic granular biomass.

**Table 4.1.** Summary of the aerobic granular biomass characteristics at the end of the experiment.

| **Parameter** | **Control** | **CF** |
|---|---|---|
| $VSS_{reactor}$ (g/L) | 7.8 | 3.4 |
| $VSS_{ef}$ (mg/L) | 100 | 150 |
| SVI (mL/g TSS) | 41 | 82 |
| Density (g VSS/$L_{granule}$) | 38 | 30 |
| Diameter (mm) | 2.3 | 5.0 |

Although the presence of coagulant-flocculant reagents seem to favour the initial aggregation of the biomass once the aerobic granules are formed it exerts a negative effect in terms of maximum biomass concentration achievable inside the SBR and of the physical properties of the obtained aggregates.

### 4.4.2. Organic matter and nitrogen removal

Since the addition of the coagulant-flocculant reagents did not suppose a significant increase in the organic matter content of the feeding, the applied OLRs to both reactors were similar and ranged between 2 and 3 kg $COD_S/(m^3 \cdot d)$. The presence of a residual amount of coagulant-flocculant reagents did not affect the organic matter removal efficiency since it was over 90% during the whole operational time for both SBR reactors (Figure 4.6).

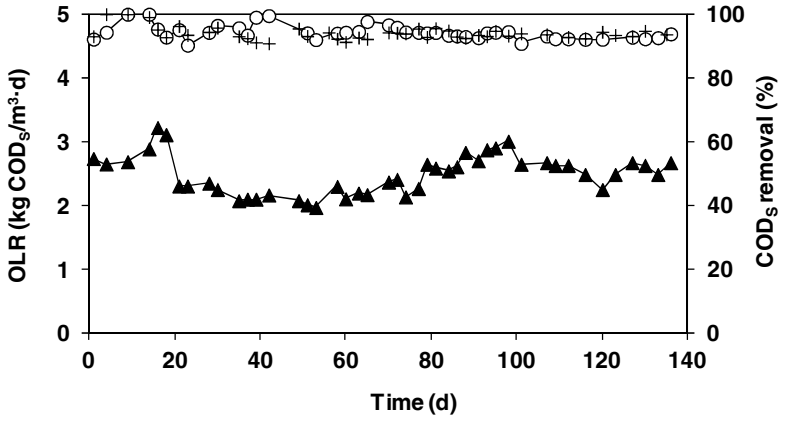


**Figure 4.6.** Applied OLR (▲) and percentage of COD removed in the control (○) and the CF (+) reactors.

The NLR fed to the SBRs varied between 0.2 and 0.3 kg $NH_4^+$-N/($m^3 \cdot d$) and the efficiency of ammonia oxidation and Total Nitrogen (TN) removal were similar in both reactors (Figure 4.7), although stable conditions were better established in the control reactor during the end part of the operational period when the higher biomass concentration was reached.

During the first six weeks of operation the TN removal was around 20-30%. Since nitrification was not detected during this period, nitrogen removal can be attributed to biomass assimilation. Ammonia oxidation was detected in both reactors around day 40 of operation. From this date nitrite was accumulated in concentrations up to 20 mg $NO_2^-$-N/L in the effluent, while the nitrate concentrations remained below 2 mg $NO_3^-$-N/L (Figure 4.7.A). Nevertheless the nitrification process was not stable and the ammonia oxidation capacity decreased in both reactors (around day 60 for control and day 70 for CF) coinciding with a decrease of the VSS inside the reactors. A problem with the aeration system was detected which avoided the correct oxygen supply for the biological process. From day 85 of operation once the air diffusers were changed the concentration of biomass inside the reactors increased, the efficiencies of ammonia

oxidation and TN removal were around 92% and 60%, respectively (Figure 4.7.B). At the end of the operational period a decrease in the VSS concentration of the CF reactor caused that the ammonia oxidation efficiency decreased from 92 to 70%, but the TN removal was maintained in the same values (60%). Therefore the reactor with coagulant-flocculant reagents presented similar efficiencies of nitrogen removal than the control reactor, but the stability of the nitrification-denitrification process was worse due to the less biomass retention capacity of the system. Luo *et al.* (2011) with a CAS system observed that for low NLRs (between 0.01 and 0.06 kg $N/m^3 \cdot d$) the obtained ammonia removal efficiency of around 98% was comparable with and without coagulant-flocculant reagent addition; however for high NLRs (0.12-0.4 kg $N/m^3 \cdot d$) they found a decrease in this efficiency obtaining the maximum of 65% for a dosage of 0.1 mg/L of coagulant. In this work with a higher concentration of coagulant (2.5 mg/L) and flocculant (1.5 mg/L) the ammonia removal efficiency was 92% at a NLR of 0.3 kg $NH_4^+$-$N/m^3 \cdot d$, which could be attributed to the fact that the biomass was aggregated in granules able to treat higher loads and with higher efficiencies than the activated sludge systems.

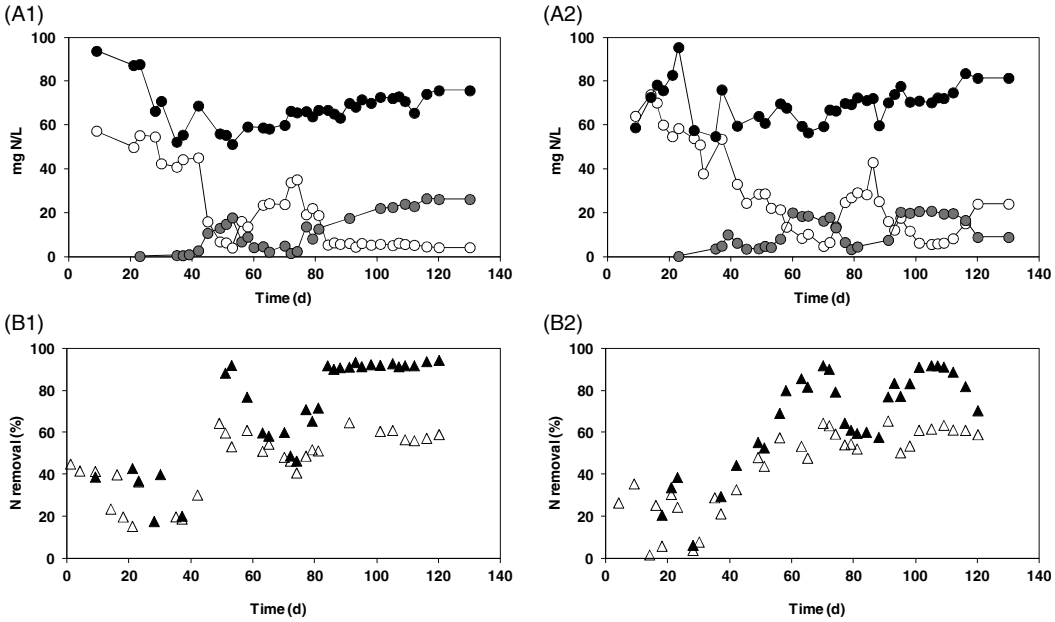


**Figure 4.7.** Control (1) and CF (2) reactors. (A) Concentration of nitrogen compounds: $NH_4^+$ influent (●), $NH_4^+$ effluent (○) and $NO_x^-$ effluent (●). (B) Percentage of $NH_4^+$ oxidation (▲) and TN removal (△).

The presence of nitrifying bacteria in the granules was detected by means of the FISH technique applied to samples of granules that were mechanically disrupted. Positive results with the probe NEU635 were obtained in both systems, indicating the presence of ammonia oxidizers belonging to the genus *Nitrosomonas* (Figure 4.8).

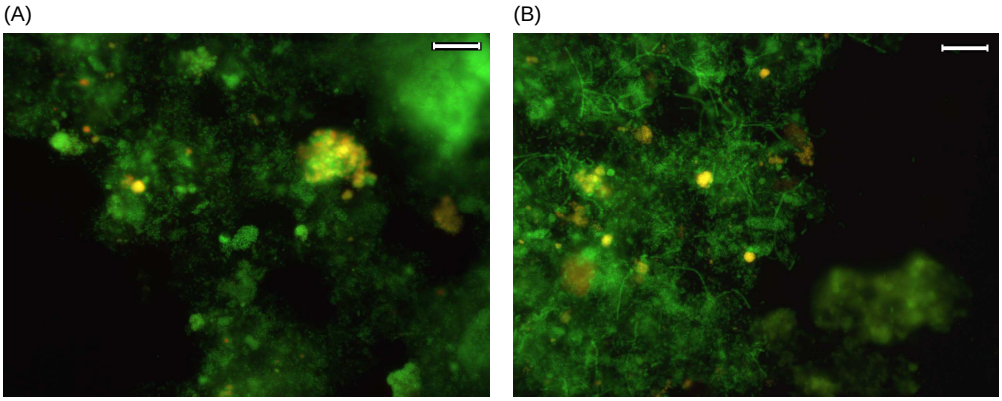


**Figure 4.8.** FISH images of $EUB338_{mix}$ (FITC-green) and NEU653 (Cy3-red) probes from (A) control and (B) CF reactors on day 135 of operation. The size bar corresponds to 25 μm.

The nitrification efficiency was closely related to the SRT value achieved in the SBRs. Nitrification efficiencies close to 100% were obtained in both systems only when the reactors operated at SRT values higher than 5 days (Figure 4.9.A). Therefore, the better stability of the nitrification process observed for the control SBR can be attributed to its higher biomass retention capacity. The maximum nitrogen removal achieved was around 60% in both systems (Figure 4.7.B). The correlation between nitrification and total nitrogen removal efficiencies reached values up to 60% (Figure 4.9.B). This fact indicates that the nitrification is the limiting step of nitrogen removal when its efficiency was lower than this value. Higher nitrification efficiencies did not improve the nitrogen removal efficiency since in that case the applied volume exchange ratio would be the limiting factor controlling the effluent quality.

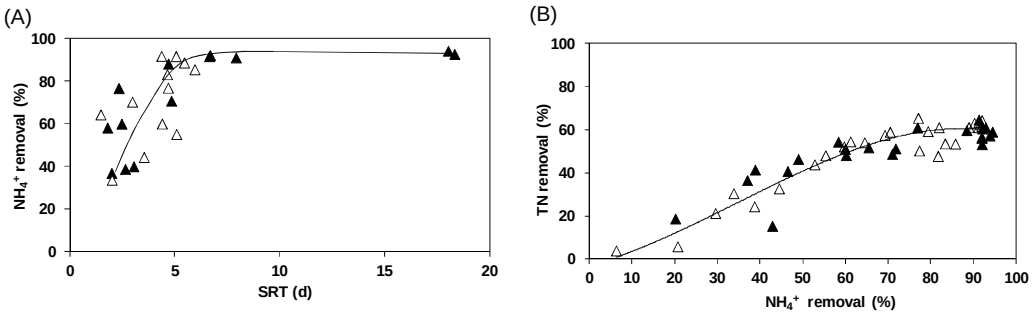


**Figure 4.9.** (A) Percentage of $NH_4^+$ oxidation as a function of the SRT and (B) relationship between TN and $NH_4^+$ removal percentages in the control (▲) and the CF (△) SBRs.

Along the whole operational period the evolution of the concentrations of the different compounds in the liquid media were measured along different cycles of operation. As an example the results obtained from a cycle measurement, performed on day 112 in both reactors,

are shown in Figure 4.10. This cycle corresponded to a period of operation considered in steady state conditions in both reactors. The obtained results indicated that the length of the feast period (presence of substrate in the liquid phase as $COD_S$) was shorter for the control (20 min) than for CF reactor (40 min). The transition from the feast to the famine period can be also followed by the profile of DO concentration in both reactors which indicates that during the feast period the DO consumption is higher than during the famine one. This longer feast phase in the case of the CF reactor provoked that the denitrification process, which took place at the beginning of the cycle when the DO concentration was lower, could prevail during a longer period. Therefore the nitrite and nitrate ($NO_x^-$) concentrations were at the end of the cycle lower than in the control reactor. The lower $NO_x^-$ concentration could also be attributed to the lower ammonia oxidation rate achieved in the CF reactor in comparison with the control one. In consequence the total nitrogen removal was similar in both reactors.

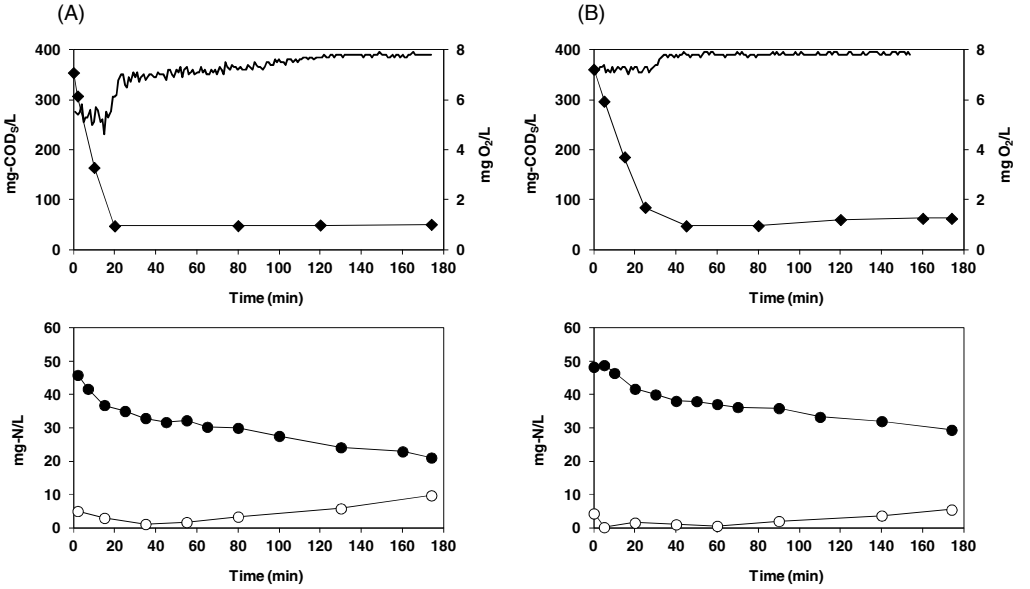


**Figure 4.10.** Profile of different compounds concentrations from a cycle measurement on day 112 in the control (A) and the CF (B) reactors: oxygen (-), $COD_S$ (◆ ), $N\text{-}NH_4^+$ (●) and $N\text{-}NO_x^-$ (○) concentrations.

The famine/feast ratio during each reactor operation was calculated by means of the COD concentration profile for the different measured cycles and represented in Figure 4.11. During the whole operational period this ratio was higher for the control than for the CF reactor, although this difference was more significant from day 80 of operation that coincides with the date when the sharp increase of biomass concentration was observed in the control reactor (Figure 4.2). At the end of the operational time the value of this ratio was of 7.6 and 3.5 for control and CF systems, respectively. Mosquera-Corral *et al.* (2005) working with a SBR using a similar cycle distribution evaluated the effect of the DO concentration on the famine/feast ratio and observed that compact and dense granules were obtained when this ratio had values

between 7.5 and 10. In the present work the presence of coagulant-flocculant reagents provoked a decrease of the famine/feast ratio in comparison with the control reactor due to the lower biomass retention capacity. To treat the same applied load by means of accumulating compounds the CF reactor needed more time due to the lower biomass concentration, which increased the feast phase and decreased the famine/feast ratio. de Kreuk and van Loosdrecht (2004) observed that a long feast phase caused a filamentous outgrowth and granule instability. Similarly in this study the presence of filamentous microorganisms in the granules surface of CF reactor (Figure 4.1.B) was detected together with the fact that the obtained aggregates presented a high average diameter and SVI value (Figure 4.2).

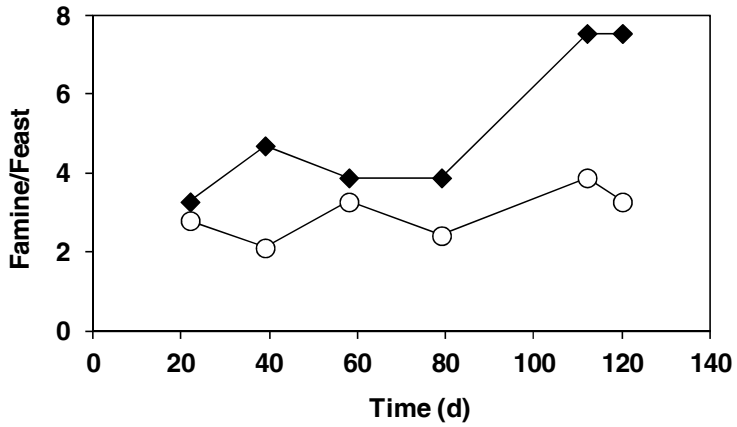


**Figure 4.11.** Famine/feast ratio during the operational period for the control (◆) and the CF reactors (○).

Although the organic matter removal is not affected by the presence of the coagulant-flocculant reagents it is important to have in mind that another biological processes such as those involved in the nitrogen removal are influenced specially by the indirect effects of these reagents. The availability of the DO influences the nitrification-denitrification processes and can be evaluated in terms of famine/feast ratio and even by performing the estimation of it consumption by means of the estimation of the SOUR.

### 4.4.3. Specific oxygen uptake rate (SOUR)

The SOUR of both reactors was periodically tested during several operational cycles to determine the impact of the coagulant-flocculant reagents on the microbial activity. The maximum specific oxygen consumption rate ($r_{max}$) was calculated according to equation [4.1] in order to avoid the effects of particle size and oxygen concentration on the specific activity obtained values. For similar SRT values, the maximum specific oxygen consumption rate observed in the control reactor was higher than that observed in the CF reactor (Figure 4.12). A similar behaviour was obtained by Lees *et al.* (2001) who studied the effect of three different coagulants with different residual ion concentrations on an activated sludge and observed a decrease in the SOUR in most of the cases. Working with aerobic granular biomass Ren *et al.*

(2008) observed that the SOUR decreased from 27 to 10 mg $O_2$/g VSS·h when the concentration of $Ca^{+2}$ in the feeding increased from 20 to 40 mg/L, suggesting that the $Ca^{+2}$ accumulation inside the granules might have a negative effect on their bioactivity. Luo *et al*. (2011) studied the effect of a trace amount of coagulant on long-term performance of activated sludge and found that the optimal dosage to improve the SOUR was 0.1 mg/L, but for higher concentrations the SOUR started to decrease. Dapena-Mora *et al*. (2007) with Anammox biomass observed a decrease of around 30% in the maximum specific activity when a flocculant concentration of 1 mg/L was applied to the system. From these results, it can be inferred that the presence of these compounds in a continuous aerobic system could decrease the biological activity when their concentration exceeds a certain value. An explanation for this effect could be the adsorption of the coagulant-flocculant compounds onto the surface of the biomass as a result of the electrostatic interaction between a positively charged group of the reagents with the negatively charged surface of the microorganisms, reducing the active surface of the biomass and consequently its bioactivity.

In the case of the control, the maximum specific oxygen consumption rate decreased from 6.0 to 2.3 g $O_2$/g VSS·d with the increase in the SRT from 2.5 to 18 d. Ouyang and Liu (2009) also observed that the bioactivity and the SOUR decreased with the increase in the SRT in a membrane bioreactor. Han *et al.* (2005) attributed this decrease to the specific biological activity at prolonged SRT by the accumulation of inert biomass due to endogenous respiration, although this is not exactly the case in the present work because the SOUR is referred to the VSS.

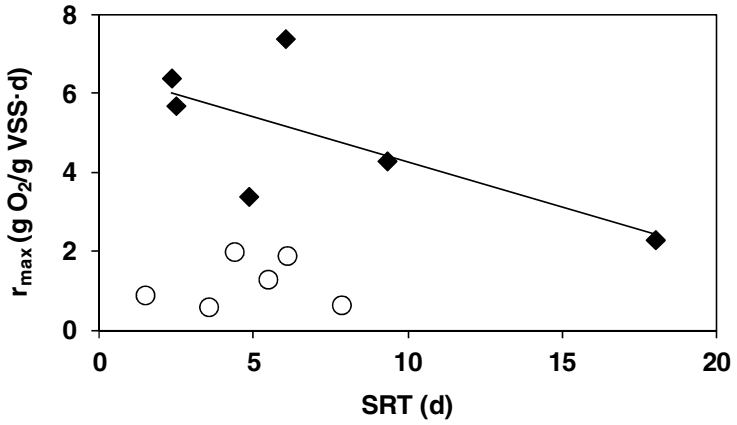


**Figure 4.12.** Maximum specific oxygen consumption rate ($r_{max}$) at different SRT values for the control (◆) and the CF (○) reactors.

The fact that the $r_{max}$ was always lower in the CF reactor than in the control one, even when the latter operated with high values of the SRT, that promote the decrease on the $r_{max}$, confirms that the presence of coagulant-flocculant reagents could decrease the microbial activity.

## 4.5. CONCLUSIONS

The aerobic granular reactor operated in the presence of trace amounts of coagulant-flocculant reagents (2.5 mg/L and 1.5 mg/L, respectively) had a lower biomass retention capacity compared to the control system due to the worse physical properties of the granules obtained. Maximum reached biomass concentrations were of 8 and 4 g VSS/L in the control and CF reactors, respectively. Granules from the CF reactor presented a fluffy and filamentous aspect which corresponded to the operational periods with low values of the famine/feast ratio observed.

In both reactors, the maximum removal efficiencies of organic matter and nitrogen achieved were similar, being around 90% and 60%, respectively. However, the operational stability of the reactor with the presence of coagulant-flocculant reagents was worse due to the low SRT obtained.

The maximum specific oxygen consumption rate ($r_{max}$) of the aerobic granules with coagulant-flocculant reagents was lower than that of the control system, which indicates that the presence of these compounds could decrease the activity of the biomass.

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# Chapter 5:

# Effects of the cycle distribution on the performance of SBRs with aerobic granular biomass


## Summary

The aerobic granular systems are mainly SBRs where the biomass is submitted to feast-famine regimes to promote its aggregation in the form of granules. In these systems different cycle distributions can be applied for the simultaneous removal of organic matter, nitrogen and phosphorus.

In Chapters 3 and 4 the development of aerobic granular biomass with an industrial and synthetic wastewater, respectively, was achieved. In both cases the same cycle distribution was applied comprising a cycle with a length of 180 minutes and a reaction phase completely aerobic according to previous works (Arrojo *et al.*, 2004; Figueroa *et al.*, 2008; Figueroa *et al.*, 2011).

In this chapter two strategies were followed in order to evaluate the effects of the cycle distribution. In a first experiment the length of the operational cycle was decreased in order to maximize the treatment capacity and consequently the famine/feast ratio also was decreased. In a second experiment, an initial anoxic phase was implemented to improve the nitrogen removal efficiency. The results obtained showed that to reduce the famine/feast ratio from 10 to 5 was possible increasing the treated Organic (OLR) and Nitrogen Loading Rate (NLR) in the system to 33%, without affecting the removal efficiencies of organic matter (97%) and nitrogen (64%) and producing a slight detriment of the granules characteristics. In another hand the implementation of an anoxic phase of 30 minutes previous to the aerobic one with a pulse-fed mode increased the nitrogen removal of pig manure from 20 to 60%, while the cycle configuration comprised by a continuous feeding simultaneous with an anoxic phase of 60 minutes did not enhance the nitrogen removal and even worsen the ammonia oxidation.

## 5.1. INTRODUCTION

Innovative technologies like those based on aerobic granular biomass constitute a promising alternative to the Conventional Activated Sludge (CAS) process for wastewater treatment. These systems are Sequencing Batch Reactors (SBR) operated in batch mode with a short initial feeding phase (pulse-fed) followed by an aerobic reaction phase that allows submitting the biomass to feast-famine regimes. These conditions are suitable to select the appropriate microorganisms to obtain compact and dense granules (de Kreuk and van Loosdrecht, 2004; Liu *et al.*, 2005; Campos *et al.*, 2009). During the feast period the microorganisms with the capacity to store Poly-Hydroxy-Alkanoates (PHA) are selected and during the famine period these storage polymers are used for growth (van Loosdrecht *et al.*, 1997). The existence of a starvation phase also induces the bacteria to become more hydrophobic, which accelerates the microbial aggregation (Bossier and Verstraete, 1996); however an excessively long famine period may not be necessary and lead to an extra energy consumption and low reactor capacity (Liu and Tay, 2007). The literature information indicates that different strategies can be applied in order to improve the performance of an aerobic granular system. In this sense some studies were focused on the establishment of the adequate cycle length to obtain a minimum famine/feast ratio (Tay *et al.*, 2002; Liu and Tay, 2008; Gao *et al.*, 2011) or on the reduction of the aeration rate in the famine period to reduce the total aeration requirement (Liu and Tay, 2006).

The modification of the cycle distribution might also have an effect on the removal efficiencies of the different pollutants. In the case of the organic matter if the length of the cycle is too short the famine/feast ratio becomes too short and it is no longer accumulated as storage compounds (PHA), but oxidized. This oxidation process requires a longer period and in some cases some incomplete organic matter removal occurs. With respect to the nutrients removal nitrogen can be also affected. It is known that in spite of the fact that the system operates during the whole reaction phase under aerobic conditions, denitrification can take place during the feast period. Due to the presence of organic matter in the liquid media the aerobic activity of the heterotrophic microorganisms provokes the depletion of oxygen in the outer layers of the granules with the subsequent occurrence of a large anoxic/anaerobic zone. The denitrification process can take place when anoxic conditions are generated in the core of the granules, nitrate is available due to the nitrification process in the outer layers of the aggregates, together with the organic matter left from the oxidation process. The ratio between aerobic and anoxic volume in the granule depends on the size of the aggregate, the Dissolved Oxygen (DO) concentration in the media and the microorganisms activity. This ratio strongly influences de nitrogen removal efficiency (de Kreuk *et al.*, 2007; Li *et al.*, 2008).

When the nitrogen removal efficiency is limited by the denitrification process, the DO concentration can be reduced to obtain an optimal ratio between aerobic and anoxic zones inside the granules or an extra anoxic phase can be incorporated to the SBR cycle (de Kreuk *et al.*, 2005b). Mosquera-Corral *et al.* (2005) in a pulse-fed granular SBR studied the effect of a decrease on the DO saturation concentration from 100% to 40% and observed the occurrence of an increase of the nitrogen removal efficiency while the biomass characteristics worsen and

the granules disintegrated. However de Kreuk *et al.* (2005a) working at low DO concentrations (40% and 20%) and with a continuous feeding along an anoxic phase previous to the aerobic one achieved an improvement in the nitrogen and phosphorus removal without affecting the properties of the aerobic granules. It must be pointed out that the former system is aimed to remove organic matter and nitrogen while the latter is operated to remove organic matter, nitrogen and phosphorous.

For all this reasons the application of a change in the cycle distribution is expected to produce changes not only in the physical properties of the biomass but also on the occurring processes.

## 5.2. OBJECTIVE

The aim of the present work is to evaluate the length and the distribution of the operational cycle which allow maximizing the treatment capacity and the nitrogen removal efficiency, respectively. In a first experiment the reduction on the famine/feast ratio needed to maintain the physical stability of granules will be determined. In a second experiment the implementation of an initial anoxic phase to improve the nitrogen removal efficiency of an aerobic granular system treating pig manure will be tested. Special attention on the possible changes of the physical characteristics of aerobic granules will be paid in both experiments.

## 5.3. MATERIALS AND METHODS

### 5.3.1. Experimental set-up and operational conditions

Three SBRs (R1, R2 and R3) of 1.8 L were operated with the same experimental set-up as the SBR described in Chapter 3 and with a different cycle distribution for each reactor comprising the following phases: feeding, reaction (anoxic/aerobic), settling and withdrawal (Table 5.1).

**Table 5.1.** Distribution of the operational cycle.

| **Phase length (min)** | **R1** | | | **R2** | **R3** |
|---|---|---|---|---|---|
| | **Stage Ia** | **Stage IIa** | **Stage IIIa** | | |
| Total cycle | 180 | 160 | 140 | 180 | 180 |
| Feeding | 3 | 3 | 3 | 3 | 60[b] |
| Anoxic phase | 0 | 0 | 0 | 30[a] | 60[b] |
| Aerobic phase | 171 | 151 | 131 | 141 | 114 |
| Settling | 1 | 1 | 1 | 1 | 1 |
| Withdrawal | 5 | 5 | 5 | 5 | 5 |

[a] An air pulse of 1 second was applied at the beginning of the cycle to homogenize the reactor content

[b] Feeding and anoxic phases took place simultaneously

### 5.3.2. Strategy of operation

***Optimization of the famine/feast ratio***

The reduction of the famine/feast ratio was tested in R1 by decreasing the length of the operational cycle. Previously to the experiments described in this chapter the reactor was operated along 300 days, being the first 140 operational days described in Chapter 4 as the "control reactor", so it contained a mature aerobic granular biomass and its operation was stable in terms of organic matter and nitrogen removal. Then the length of the operational cycle was progressively reduced from 180 minutes (Stage Ia) to 140 minutes (Stage IIIa), which supposed an increase of the OLR and NLR applied to the system (Table 5.2). R1 was fed with a synthetic medium with the same composition as described in Chapter 4.

**Table 5.2.** Operational conditions for each stage of operation of R1.

| Parameter | Stage Ia | Stage IIa | Stage IIIa |
|---|---|---|---|
| Days of operation | 300-352 | 353-386 | 387-424 |
| Cycles per day | 8 | 9 | 10 |
| HRT (d) | 0.250 | 0.225 | 0.200 |
| NLR (kg $NH_4^+$-N/$m^3$·d) | 0.182 | 0.208 | 0.244 |
| OLR (kg $COD_S$/$m^3$·d) | 2.466 | 2.836 | 3.280 |

***Optimization of nitrogen removal efficiency***

In order to improve the nitrogen removal efficiency the implementation of an initial anoxic phase to the cycle of operation was tested in two laboratory SBRs (R2 and R3). The lengths of the anoxic phase and the feeding pattern were different in both reactors. R2 was fed in a pulse mode (3 minutes) followed by one air pulse of 1 second to homogenize the bulk liquid and an anoxic phase of 30 minutes without mixing. R3 was fed during 60 minutes from the bottom of the reactor to maintain a plug flow regime during the initial anoxic phase. The Hydraulic Retention Time (HRT) was of 0.25 days and the number of cycles per day was of 8. Both reactors were fed with swine manure collected from a pig farm located in Santiago de Compostela (Spain). Previously to be fed to the reactors the raw manure was settled to remove as much as possible the solid fraction and stored at 4 ºC to avoid degradation processes. R2 and R3 were operated for 70 and 42 days, respectively, following a strategy of OLR progressive increase. The operational period was divided in three stages according to the batches of pig manure picked up in the farm (Table 5.3).

Both SBRs were inoculated with mature aerobic granular biomass from a SBR pilot plant (100 L) in operation and treating the same substrate (pig manure) (Morales *et al.*, 2011). The biomass presented the following physical characteristics: 11.4 g TSS/L, 10.8 g VSS/L, a SVI of 36 mL/g TSS and an average particle size of 2.52 mm. In the SBR pilot plant the cycle of operation was similar to the cycle used in R1-Stage Ia (with a complete aerobic phase and a total length of 180 minutes) and distributed as follows: feeding 7 minutes, aerobic reaction 168

minutes, settling 3 minutes and effluent withdrawal 2 minutes (Jungles *et al.*, 2011). When the inocula were collected the Pilot Plant was working with an OLR around 4 kg $COD_S/m^3 \cdot d$ and a removal efficiency of 75%, while the applied NLR was of 1 kg $NH_4^+$-$N/m^3 \cdot d$ with a removal efficiency of 75% for ammonia and 20% for TN.

**Table 5.3.** Composition of the feeding and applied nutrient loading rates to R2 and R3.

| Parameter | Stage Ib | Stage IIb | Stage IIIb |
|---|---|---|---|
| Days of operation (R2) | 0-19 | 20-34 | 35-70 |
| Days of operation (R3) | 0-19 | 20-34 | 35-42 |
| $COD_T$ (mg/L) | 886 ± 9 | 1323 ± 12 | 1846 ± 48 |
| $COD_S$ (mg/L) | 532 ± 18 | 760 ± 35 | 1222 ± 126 |
| $NH_4^+$-N (mg/L) | 306 ± 15 | 126 ± 5 | 243 ± 32 |
| $PO_3^-$-P (mg/L) | 0.33 ± 0.02 | 0.07 ± 0.05 | 0.35 ± 0.20 |
| COD:N ratio (g/g) | 1.74 | 6.03 | 5.03 |
| TSS (g/L) | 0.24 ± 0.08 | 0.25 ± 0.09 | 0.27 ± 0.11 |
| VSS (g/L) | 0.22 ± 0.08 | 0.23 ± 0.08 | 0.26 ± 0.10 |
| pH | 7.78 ± 0.01 | 7.58 ± 0.01 | 7.54 ± 0.07 |
| OLR (kg $COD_S/ m^3 \cdot d$) | 2.13 ± 0.07 | 3.04 ± 0.14 | 4.89 ± 0.50 |
| NLR (kg $NH_4^+$-$N/m^3 \cdot d$) | 1.22 ± 0.06 | 0.50 ± 0.08 | 0.97 ± 0.13 |

### 5.3.3. Analytical methods

Ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$), DO, pH, total suspended solids (TSS), volatile suspended solids (VSS) concentrations and Sludge Volume Index (SVI) were determined according to the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The phosphate ($PO_4^{3-}$) was determined by ion chromatography. Chemical Oxygen Demand (COD) was determined by a semi-micro method (Soto *et al.*, 1989); total COD ($COD_T$) was measured directly in the sample and the soluble COD ($COD_S$) from the sample filtered through 0.45 μm pore size filters. 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). Biomass density in terms of g VSS per litre of granules, was determined with dextran blue following the methodology proposed by Beun *et al.* (1999). The PHA concentration was measured according to a modification of the methodology proposed by Pijuan *et al.* (2005).The biomass composition (C, H, N, O and S) was determined with an elemental analysis technique based on the complete and instantaneous oxidation (combustion) of the sample and the further quantification of the gases from the combustion through a thermal conductivity detector (model CHNS FISONS EA 1108 for C, H, N and S; model CARLO ERBA EA 1108 for oxygen).

The denitrification activity of the biomass was determined by means of batch experiments according to the methodology proposed by Buys *et al.* (2000) in order to know the biomass denitrifying capacity on PHA as organic matter substrate.

### 5.3.4. Calculations

***Carbon balances***

The concentration of acetate as C mmol/L in R1 was calculated assuming that the COD measured in the reactor was only due to this compound.

The concentration of biomass as C mmol/L was calculated using the stoichiometric composition of a biomass sample collected from the reactor and measured by elemental analysis which corresponded to: $CH_{1.77}N_{0.20}O_{0.59}S_{0.004}$.

The amount of PHA as Poly-Hydroxy-Butyrate (PHB) contained in the reactor at a certain moment, the specific PHB synthesis rate ($q_{PHB}$) and the specific acetate uptake rate ($-q_{Ac^-}$) were calculated according to Beun *et al.* (2000) assuming that they follow a zero-order kinetic and a constant biomass concentration along a cycle of operation.

***Nitrogen balances***

Nitrogen balances were calculated following the procedures described in Chapter 2.

***Consumption and production rates of the nitrogen compounds***

The specific ammonia and $NO_x^-$ consumption rates ($-q_{NH4+}$, $-q_{NOx^-}$) and the specific $NO_x^-$ production rate ($q_{NOx^-}$) were calculated according to Mosquera-Corral *et al.* (2005).

## 5.4. RESULTS AND DISCUSSION

### 5.4.1. Optimization of the famine/feast ratio

***Aerobic granules characteristics***

The reactor R1, already in operation during 300 days previously to the start of the present experiment, presented at the beginning of Stage Ia a stable aerobic granular biomass with a concentration of 8.7 g VSS/L and a mean particle size of 1.0 mm. The settling characteristics of the aerobic granules were suitable being the SVI low of 45 mL/g TSS and the biomass density high of 69 g $VSS/L_{granule}$, which allowed an appropriated biomass retention inside the reactor and produced low solids concentration in the effluent (93 mg VSS/L) (Table 5.4).

**Table 5.4.** Properties of the aerobic granular biomass from R1.

| Parameter | Stage Ia | Stage IIa | Stage IIIa |
|---|---|---|---|
| SRT | 21 ± 7 | 18 ± 5 | 8 ± 5 |
| $VSS_{reactor}$ (g/L) | 8.7 ± 2.2 | 6.5 ± 1.2 | 5.6 ± 1.1 |
| $VSS_{effluent}$ (mg/L) | 93 ± 36 | 145 ± 95 | 234 ± 76 |
| F/M ratio (g $COD_S$/g VSS·d) | 0.31 ± 0.09 | 0.46 ± 0.10 | 0.62 ± 0.10 |
| Granule diameter (mm) | 1.0 ± 0.2 | 1.4 ± 0.3 | 2.3 ± 0.6 |
| SVI (mL/g TSS) | 45 ± 13 | 46 ± 3 | 60 ± 14 |
| Density (g $VSS/L_{granule}$) | 69 ± 12 | 52 ± 7 | 44 ± 11 |

During Stages IIa and IIIa the increase of the number of cycles per day, associated with the progressive shortening of the cycle length (Table 5.2), caused an increase of the biomass wash out and the consequent decrease of solids concentration inside the reactor from 8.7 to 5.6 g VSS/L (Table 5.4). This fact also involved a reduction in the Solids Retention Time (SRT) from 21 days (Stage Ia) to 8 days (Stage IIIa). The Food to Microorganism ratio (F/M) increased associated with the reduction on the solids concentration and the increase on the treated OLR. As it was discussed in previous Chapters 3 and 4, values of the F/M ratio lower than 1 g $COD_S$/g VSS·d are necessary to maintain aerobic granules with good settling properties. In this study the ratio value increased from 0.31 (Stage Ia) to 0.62 g $COD_S$/g VSS·d (Stage IIIa), but it maintained below this critical level. Although the settling properties of the biomass changed slightly: the SVI remained in a similar value in Stage IIa but then it increased from 46 to 60 mL/g TSS in Stage IIIa, while the density decreased from 69 g $VSS/L_{granule}$ (Stage Ia) to 44 g $VSS/L_{granule}$ (Stage IIIa). The mean particle size of the aerobic granules increased gradually from 1.0 to 2.3 mm, what is in agreement with the results found by Liu and Tay (2008). These authors observed that the shorter the famine period the higher the size of the granules. The volume size distributions for Stages Ia and IIIa can be compared in Figure 5.1. The granules size distribution in Stage Ia ranged between 0.6 and 1.8 mm, with a contribution of 71% concentrated on particles in the range 0.8-1.2 mm. However the volume size distribution in Stage IIIa was more dispersed and the granules diameter ranged between 0.4 and 5.0 mm.

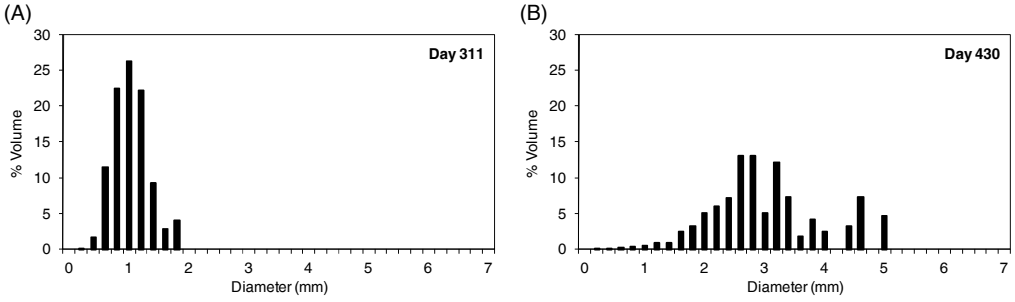


**Figure 5.1.** Volume size distribution of the aerobic granules (%): (A) Stage Ia and (B) Stage IIIa.

The aspect of the aerobic granules also changed with the reduction in the cycle length. During Stage Ia the aerobic granules presented a smooth surface and a small average particle size (Figure 5.2.A), that did not change along the Stage IIa (Figure 5.2.B). However in Stage IIIa the size of the granules increased and filamentous structures appeared on their surfaces (Figure 5.2.C). This filamentous outgrowth was also observed by de Kreuk and van Loosdrecht (2004) when a long feast phase was applied (reduction of the famine/feast ratio) and by Adav *et al.* (2009) after the increase of the treated OLR. In this work both effects occurred in Stage IIIa, the reduction on the famine/feast ratio and the increase of the treated OLR, which justify the filamentous outgrowth on the granules surface.

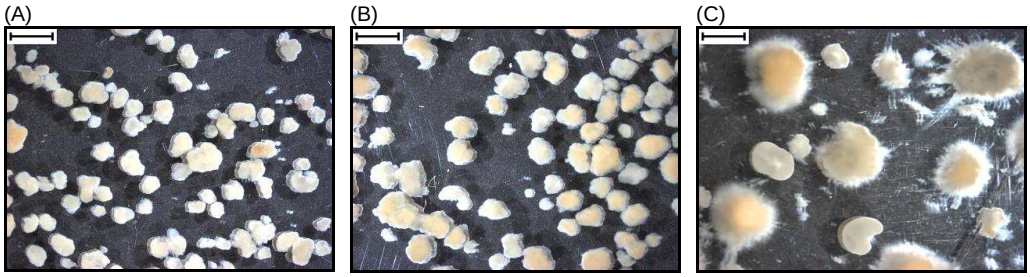


**Figure 5.2.** Images of aerobic granular biomass from R1: (A) Stage Ia, (B) Stage IIa and (C) Stage IIIa. The size bar corresponds to 2 mm.

From the previous results it can be inferred that the physical properties of the biomass are changed due to the cycle length modification, which can also affect the performance of the different processes taking place in the reactor.

***Organic matter and nitrogen removal***

The reduction of the cycle length, which involved an increase of the applied OLR and NLR to the system of 33% did not affect the removal efficiencies of organic matter and nitrogen, which were maintained along the three stages of operation around 97% and 64%, respectively (Table 5.5). The ammonia oxidation worsened a little in Stage IIIa respect to the previous stages. Nitrogen balances were calculated for each stage 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 5.5).

**Table 5.5.** Organic and nitrogen removal efficiencies during the different stages for R1.

| Parameter | Stage Ia | Stage IIa | Stage IIIa |
|---|---|---|---|
| COD removal (%) | 97 ± 1 | 98 ± 1 | 96 ± 1 |
| $NH_4^+$ oxidation (%) | 96 ± 5 | 100 ± 1 | 87 ± 14 |
| TN removal (%) | 63 ± 5 | 64 ± 6 | 64 ± 8 |
| $N_{assimilated}$ (%)[a] | 55 | 47 | 49 |
| $N_{denitrifed}$ (%)[a] | 45 | 53 | 51 |

[a] Calculated as the percentage respect to the TN removal.

In order to determine the famine/feast ratio the concentration of acetate was monitored along the operational cycles measured during the different stages, an example is presented in Figure 5.3.

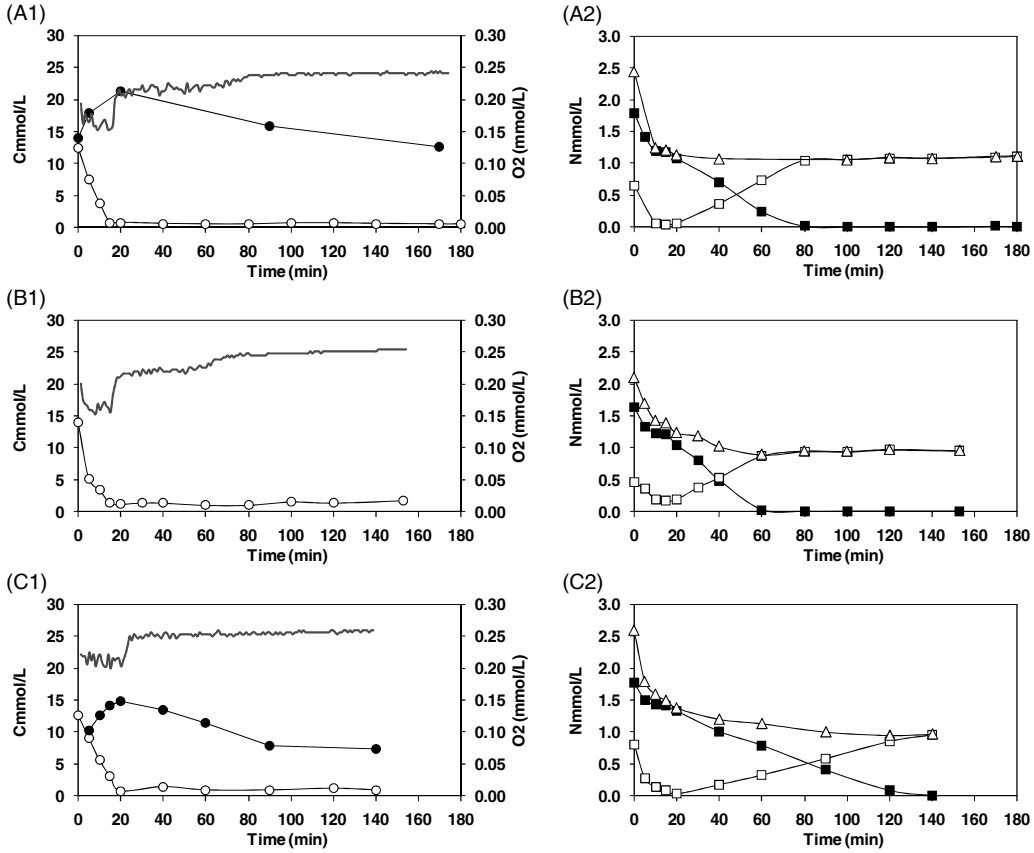


**Figure 5.3.** Concentration profiles during operational cycles of R1: PHB (●), acetate (○), dissolved oxygen (-), TN (△), $NH_4^+$ (■) and $NO_X^-$ (□). (A) Stage Ia (day 323), (B) Stage IIa (day 359) and (C) Stage IIIa (day 408).

The analysis of the composition storage compounds accumulated inside the biomass as PHA indicated that the polymer present was basically PHB. This is in accordance with another works that used acetate as organic carbon source in a system operated with a feast-famine regime in aerobic conditions (Salehizadeh and Van Loosdrecht, 2004). The profile of the PHB concentration was similar during Stages Ia and IIIa (Figure 5.3; data not available for Stage II) and showed that during the feast phase part of the consumed acetate was accumulated as storage materials (PHB) and then, during the famine phase, the growth of the aerobic granules took place on the stored PHB after depletion of external organic carbon source (acetate).

The PHB content expressed as concentration inside the reactor at the end of the feast phase was lower in Stage IIIa (15 $Cmmol_{PHB}/L$) than in Stage Ia (21 $Cmmol_{PHB}/L$) due to the

lower biomass concentration in the former than in the latter. However, if the concentration of storage compounds is referred to the biomass, the value obtained in Stage IIIa (0.063 $Cmol_{PHB}/Cmol_{biomass}$) was higher than that obtained in Stage Ia (0.052 $Cmol_{PHB}/Cmol_{biomass}$), which would indicate an increase of the biomass capacity to store PHB. Furthermore the value of the F/M ratio in Stage IIIa was higher than in Stage Ia (Table 5.4), which indicates that the quantity of substrate available for microorganism was higher and, consequently, the quantity of substrate susceptible to be accumulated too. An estimation of the PHB consumed during the starvation period shows that in Stage IIIa this consumption was higher (0.032 $Cmol_{PHB}/Cmol_{biomass}$) than in Stage Ia (0.021 $Cmol_{PHB}/Cmol_{biomass}$), so the shorter duration of the famine phase did not imply a lower consumption. During Stage Ia when the SRT value was of 21 days, the ratio of organic matter stored inside the bacteria cells as PHB from the consumed acetate ($q_{PHB}/-q_{Ac-}$) was of 0.48 $Cmol_{PHB}/Cmol_{Ac-}$ (Table 5.6); however in Stage IIIa with a SRT of 7 days this value was slightly higher (0.52 $Cmol_{PHB}/Cmol_{AC-}$). A similar behaviour was observed by Chua *et al.* (2003) with activated sludge. These authors obtained a PHA production capacity 10% higher with a SRT of 3 days than with a SRT of 10 days. They explain that with the shorter SRT a microbial community with large PHA production capacity may be selected and also that the amount of the inert fraction of the biomass is low. Jiang *et al.* (2011) established that the enrichment of biomass with the capacity to store a high fraction of PHB could be achieved minimizing the number of cycles per SRT. In this work although the number of cycles increased in each stage the SRT decreased simultaneously, so for Stage IIIa the number of cycles per SRT was lower than during Stage Ia.

**Table 5.6.** Specific production and consumption rates obtained in each of the different cycles.

| Parameter | Stage Ia | Stage IIa | Stage IIIa |
|---|---|---|---|
| Famine/Feast ratio[a] | 9.5 ± 1.5 | 7.8 ± 1.8 | 4.9 ± 0.8 |
| Day of operation | 323 | 359 | 408 |
| SRT (d) | 21 | 10 | 7 |
| $-q_{Ac-}$ ($Cmol_{Ac-}/Cmol_{biomass}\cdot h$) $\cdot 10^3$ | 114.09 | 281.91 | 151.18 |
| $q_{PHB}$ ($Cmol_{PHB}/Cmol_{biomass}\cdot h$)$\cdot 10^3$ | 54.39 | - | 78.60 |
| $q_{PHB}/-q_{Ac-}$ (Cmol/Cmol) | 0.48 | - | 0.52 |
| $-q_{NH4+}$ ($Nmol_{NH4+}/Cmol_{biomass}\cdot h$) $\cdot 10^3$ | 2.63 | 8.85 | 3.17 |
| $-q_{NOx-}$ ($Nmol_{NOx-}/Cmol_{biomass}\cdot h$) $\cdot 10^3$ | 8.79 | 9.04 | 12.34 |
| $q_{NOx-}$ ($Nmol_{NOx-}/Cmol_{biomass}\cdot h$) $\cdot 10^3$ | 2.41 | 5.19 | 2.09 |
| $q_{NOx-}/-q_{NH4+}$ (Nmol/Nmol) | 0.92 | 0.59 | 0.66 |

[a] Determined as the average value of different operational days along the corresponding stage.

Although the TN removal efficiency was similar during the different stages the profile of the nitrogenous compounds along the operational cycle varied due to the reduction in the famine/feast ratio (Figure 5.3).

During Stage Ia the ammonia oxidation was completed after 80 minutes with a specific ammonia consumption rate ($-q_{NH4+}$) of $2.63 \cdot 10^{-3}$ $Nmol_{NH4+}/Cmol_{biomass} \cdot h$ (Table 5.6), while the nitrite and nitrate ($NO_x^-$) were produced along the famine period until the ammonia was fully depleted with a specific $NO_X^-$ production rate ($q_{NOx-}$) of $2.41 \cdot 10^{-3}$ $Nmol_{NOx-}/Cmol_{biomass} \cdot h$. So the $NO_X^-$ compounds accumulated respect to the ammonia oxidation ($q_{NOx-}/-q_{NH4+}$) in a ratio of 0.92, which indicates that the denitrification along the famine phase was negligible. The $NO_x^-$ compounds were removed in the first 15 minutes of the next cycle via denitrification along the feast phase, with a specific $NO_X^-$ consumption rate ($-q_{NOx-}$) of $8.79 \cdot 10^{-3}$ $Nmol_{NOx-}/Cmol_{biomass} \cdot h$, due to the presence of acetate as organic carbon source and to the oxygen consumption in the outer part of the granules by the aerobic heterotrophic bacteria (Figure 5.3.A1). The balance performed to all the nitrogenous compounds shows that the TN removal took place only along the feast phase: the 55% was used for bacterial growth and the rest was removed via nitrification-denitrification (Table 5.5).

In Stage IIa, with the reduction of the famine/feast ratio from 9.5 to 7.8, the profile of nitrogenous compounds experienced a slight variation (Figure 5.3.B2). The $NH_4^+$ required less time to be oxidised (60 minutes) with a higher $-q_{NH4+}$ value ($8.85 \cdot 10^{-3}$ $Nmol_{NH4+}/Cmol_{biomass} \cdot h$) than previous stage (Table 5.6). The denitrification at the end of the feast phase was not complete, although the value of $-q_{NOx-}$ was similar to that obtained in Stage Ia. The profile of TN indicated that its removal occurred not only along the feast phase (first 20 minutes of the cycle) but also along the beginning of the famine phase (between 20-60 minutes of the cycle). The $q_{NOx-}/-q_{NH4+}$ ratio was of 0.59, so the denitrification along the famine phase was more favoured than in Stage Ia. The TN removal balance indicated that the 53% was eliminated via nitrification-denitrification, while the 47% was for assimilation (Table 5.5).

In the case of Stage IIIa the reduction of the famine/feast ratio down to 4.9 caused a decrease of biomass concentration inside the reactor and the $NH_4^+$ required the total time of the cycle to be oxidized (Figure 5.3.C2) with a $-q_{NH4+}$ value of $3.17 \cdot 10^{-3}$ $Nmol_{NH4+}/Cmol_{biomass} \cdot h$ (Table 5.6). The $NO_x^-$ compounds were removed via denitrification along the feast phase (similarly as in Stages Ia and IIa) but also along the total famine phase, as it could be observed in the decreasing profile of TN in the aforementioned figure. During this stage the $q_{NOx-}/-q_{NH4+}$ ratio was of 0.66 and the percentage of nitrogen denitrified was of 51%, which indicated that the denitrification was more favoured than in Stage Ia but a little worse than in Stage IIa.

With regards to Stages IIa and IIIa, the removal of TN during the famine phase could be related to the capacity of the aerobic granular biomass to use the internal stored PHB as carbon source (Qin *et al.*, 2005). In order to check this hypothesis, the denitrification capacity of the aerobic granular biomass with the internal PHB as carbon source was determined by means of denitrification activity batch tests. The maximum denitrifying activity measured with the granular biomass based on PHB was of $8.3 \cdot 10^{-3}$ $Nmol_{NOx-}/Cmol_{biomass} \cdot h$, that is a value higher than that obtained by Quin *et al.* (2005) of $2.1 \cdot 10^{-3}$ $Nmol_{NOx-}/Cmol_{biomass} \cdot h$. The denitrification along the famine phase was also possible due to the higher diameter of the granules in Stages IIa and IIIa than in Stage Ia, so the oxygen penetration depth corresponded to a small part of the total volume of the granules (Li *et al.*, 2008) and also that the ammonia oxidation process consumed

part of this oxygen in the outer layers. Both conditions allowed that a large volume of the granules was anoxic. In Stage IIIa the 6.8% of the consumed PHB along the famine phase was estimated to be used as source of carbon for denitrification which explained the high consumption of this compound observed (data nor available for Stage IIa).

Therefore although the reduction on the famine/feast ratio did not affect the TN removal the profile of the nitrogen compounds changed and with them the processes involved. The denitrification process occurred along the feast phase independently of the famine/feast ratio. However the denitrification process along the famine phase was improved with the reduction of the famine/feast ratio, together with the increase of the capacity to store PHB. The optimal famine/feast ratio to enhance the denitrification process was found to be 7.8. For values of the famine/feast ratio lower than 4.9 a worse reactor performance in terms of nitrogen removal is expected, since this is the minimum observed ratio necessary to achieve complete ammonia oxidation (Figure 5.3.C2).

### 5.4.2. Optimization of the nitrogen removal

***Aerobic granules characteristics***

The different feeding and reaction patterns imposed to each reactor were evaluated in terms of the changes in the biomass physical properties. At the beginning of the experiment since R2 and R3 were inoculated with the same source and amount of aerobic granular biomass they presented identical solids concentration and SVI values (Figure 5.4). Then, along the operational period, the solids concentration inside R2 fluctuated between 8 and 12 g VSS/L, while in R3 it remained less variable with values between 9 and 11 g VSS/L. The SVI increased in R2 from 30 to 54 mL/g TSS (Stage Ib), but then it decreased along Stages IIb and IIIb down to 23 mL/g TSS. In R3 the SVI slightly decreased along the whole operational time and the obtained values were lower than in R2.

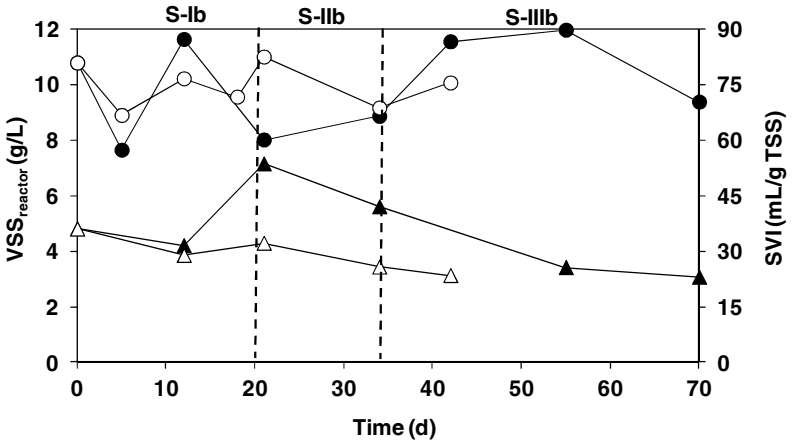


**Figure 5.4.** Evolution of VSS concentration in R2 (●) and R3 (○); and SVI value in R2 (▲) and R3 (△).

The initial average diameter of the granules was of 2.5 mm and then the size of the aggregates increased in both reactors until values of 4.1 and 3.6 mm in R2 and R3, respectively. This increase in the particle size might be related to the different hydrodynamic stress in the laboratory reactors respect to the Pilot Plant origin of the biomass. Although the applied superficial upflow air velocity was the same in the laboratory reactors and in the Pilot Plant (2.4 cm/s) the incorporation of an anoxic phase without agitation involved a reduction in the time of the application of the shear stress. Figure 5.5 shows that the granules from R2 appeared more compact and their surface smoother than those of R3, probably due to the longer application of the shear stress occurring in the former (Tay and Liu; 2001).

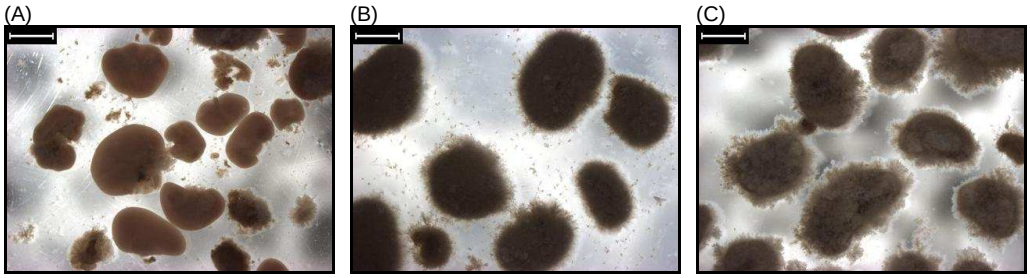


**Figure 5.5.** Images of the aerobic granular biomass: (A) inoculum, (B) R2 and (C) R3. The size bar corresponds to 2 mm.

***Organic matter removal***

The organic matter removal efficiency in Stage Ib was around 60 and 40% for R2 and R3, respectively (Figure 5.6). This low efficiency could be due to the high fraction (40-60%) of a slowly or non biodegradable fraction of the pig manure (Shin *et al.*, 2005). Then, during Stages IIb and IIIb when a new batch of feeding media was used, the organic matter removal increased up to around 75% in both reactors, even if the treated OLR was higher than in Stage Ib. Reactors R2 and R3 presented similar removal efficiencies of 75% as those obtained in the Pilot Plant, origin of the sludge inoculated in R2 and R3, with a reaction phase in aerobic conditions, where the treated OLR was around 4 kg COD/$m^3$·d (Morales *et al.*, 2011).

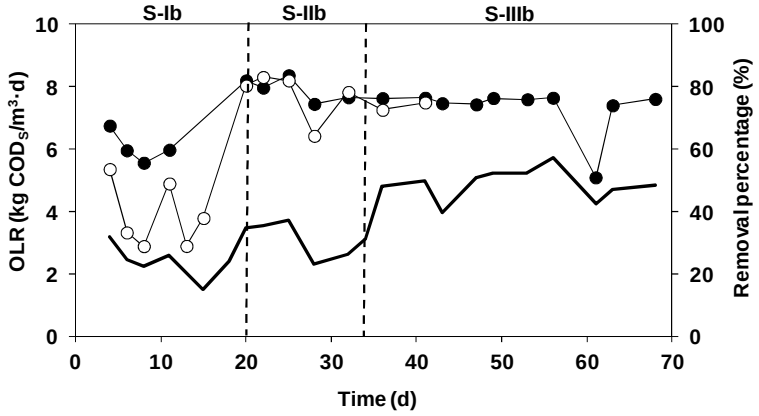


**Figure 5.6.** Evolution profiles of the applied OLRs (**–**) and removal efficiencies in R2 (●) and R3 (○).

The evolution of the organic matter concentration along one operational cycle was measured in different days of operation to determine the length of the feast phase. The profiles obtained on day 37 for R2 and R3 are presented in Figure 5.7 and compared with that of the Pilot Plant, source of inoculum and considered the reference system. The consumption of $COD_S$ took place along the anoxic and the aerobic phases for R2, while in R3 only along the aerobic one. The percentage of organic matter removal during the anoxic phase of R2 was of 16% and due to the consumption by the denitrification process. Then along the aerobic phase the feast period lasted 20 minutes (from 30 to 50 minutes), similar to that in the Pilot Plant. However in R3 the consumption of organic matter did not occur along the anoxic phase and the feast period on the aerobic one was longer than that of the Pilot Plant and of R2 lasting 50 minutes (from 60 to 110 minutes). The famine/feast ratio was of 11.0, 6.5 and 1.4 for the Pilot Plant, R2 and R3, respectively.

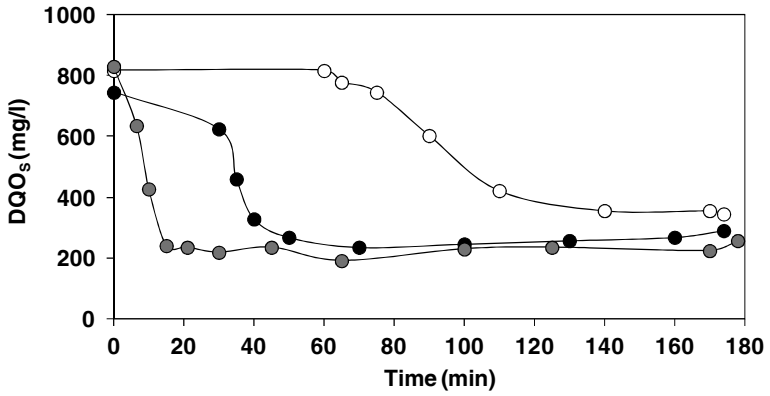


**Figure 5.7.** Concentration profiles of $COD_S$ along a cycle of operation in the Pilot Plant (●), R2 (day 37) (●) and R3 (day 37) (○).

***Nitrogen removal***

The applied NLR in both reactors was the same along the whole operational period (Figure 5.8). In Stage Ib it remained between 1.0 and 1.5 kg $NH_4^+$-N/$m^3$·d, but in Stage IIb it was only around 0.5 kg $NH_4^+$-N/$m^3$·d due to the low concentration of $NH_4^+$ in the feeding. Then in Stage IIIb it increased again to around 1.0 kg $NH_4^+$-N/$m^3$·d. The first days of operation the percentage of $NH_4^+$ oxidized and TN removal were around 50% and 25%, respectively, in both reactors. These values compared with the efficiencies obtained in the Pilot Plant (Morales *et al.*, 2011) indicated a reduction in the ammonia oxidizing capacity, although the TN removal was maintained similar.

From day 10 of operation the $NH_4^+$ oxidation and TN removal efficiencies in R2 started to increase up to 80% and 60%, respectively. On day 40 the TN removal efficiency started to decrease and achieved a value of 40% at the end of the operational period. This decrease in the

nitrogen removal was associated with the reduction of the SRT inside the reactor (from 16 to 7 d on days 35 and 70 of operation, respectively).

In R3 the $NH_4^+$ oxidation and the TN removal efficiencies decreased from day 10 of operation, being around 20-30% in Stage IIb and around 15% in Stage IIIb. Due to this low nitrogen removal efficiency the reactor was stopped on day 42 of operation.

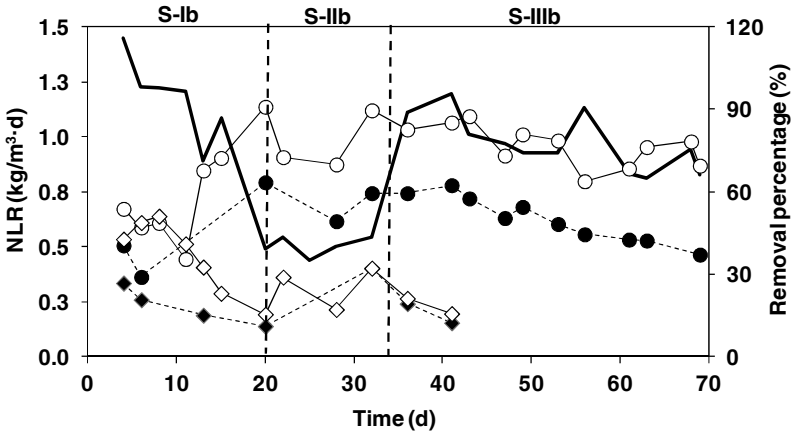


**Figure 5.8.** Profiles of applied NLR (-), efficiency of $NH_4^+$ removal in R2 (○) and R3 (◇) and efficiency of TN removal in R2 (●) and R3 (◆).

To know the processes that took place along a cycle of operation the concentrations of the different nitrogenous compounds were monitored and compared with those obtained in the Pilot Plant, source of inoculum and considered the control (Figure 5.9).

In the Pilot Plant, with a complete aerobic phase, the denitrification at the beginning of the cycle was favoured by the low penetration of the DO in the feast phase (Li *et al.*, 2008). In this way the nitrite and nitrate accumulated at the end of the previous cycle were completely degraded, but the $NO_x^-$ compounds were accumulated along the whole famine phase (from minute 20) due to the ammonia oxidation, without TN removal.

In R2 the denitrification took place along the anoxic phase at the beginning of the cycle (0-30 minutes) and also during the feast period of the aerobic phase (30-50 minutes). Furthermore in R2 only $NO_2^-$ was accumulated along the rest of the cycle, while in the Pilot Plant $NO_3^-$ was also formed. The TN removal took place along the whole cycle of operation. In R3 the ammonia oxidation and TN removal were very low with a slight change in the profile along the whole cycle.

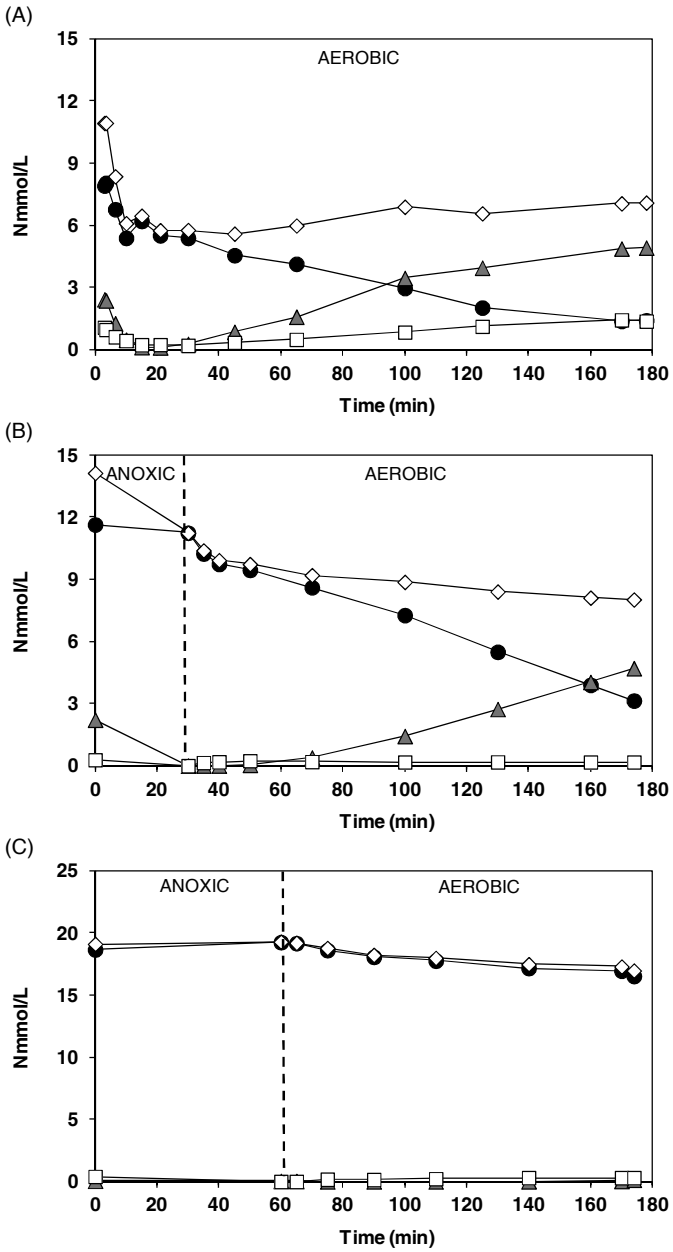


**Figure 5.9.** Concentrations profiles of TN (◇), $NH_4^+$ (●), $NO_2^-$ (▲) and $NO_3^-$ (□) along a cycle of operation in (A) Pilot Plant, (B) R2 (day 37) and (C) R3 (day 37).

In order to compare the nitrogen removal efficiency between this work and the reactor of origin in Table 5.7 the values treating the same NLR and with similar solids concentration are

presented. A rough balance calculation indicated that the cycle configuration with a previous anoxic phase and a fed-pulse mode (R2) supposed an enhancement of the nitrogen removal from 20% (Pilot Plant) to 60% and a reduction in the operational costs associated to the aeration energy. The application of mechanical mixing along the anoxic phase was not necessary due to the fact that the nitrogen gas, produced during the denitrification, created a circulating movement on the liquid. The cycle configuration with a previous anoxic phase and a simultaneous feeding in plug-flow mode (R3) did not enhance the nitrogen removal and even the ammonia oxidation worsened from 75% (Pilot Plant) to 20%, probably due to the low value of the famine/feast ratio (1.4) on this system. The concentration of nitrogen assimilated in R2 and R3 was similar (around 20 mg/L), but respect to the TN removed it represented the 10 and 46% in R2 and R3, respectively. Therefore from the TN removed in R2 the 90% was by the nitrification-denitrification process.

**Table 5.7.** Comparison of nitrogen removal percentages between the Pilot Plant, R2 and R3.

| **Parameter** | **Pilot Plant** [a] | **R2** [b] | **R3** [b] |
|---|---|---|---|
| $VSS_{reactor}$ (g/L) | 11 | 9 | 9 |
| SRT (d) | 17 | 16 | 13 |
| Average diameter (mm) | 2.5 | 4.1 | 3.6 |
| NLR (kg $N/m^3 \cdot d$) | 1.1 | 1.1 | 1.1 |
| $NH_4^+$ removal (%) | 75 | 80 | 20 |
| TN removal (%) | 20 | 60 | 20 |
| $N_{assimilated}$ (%) | ND | 10 | 46 |
| $N_{denitrifed}$ (%) | ND | 90 | 55 |

[a] Day of operation 140, when the aerobic granules were taken to inoculate R1 and R2.
[b] Day of operation 35.
ND: Not Data

## 5.5. CONCLUSIONS

The reduction in the famine/feast ratio from 10 to 5, allowed increasing the OLR and NLR treated without affecting the removal efficiencies of organic matter (97%) and nitrogen (64%) and with a slight detriment on the physical properties of aerobic granular biomass.

The reduction of the famine/feast ratio from 10 to 5 increased the capacity of the system to accumulate storage compounds (PHB) and this benefitted the occurrence of the denitrification process along the famine phase based on the consumption of these storage compounds.

The implementation of an anoxic phase before the aerobic one with a pulse-fed mode improved the nitrogen removal of pig manure from 20 to 60% respect to the cycle configuration with a complete aerobic phase. The cycle configuration with an anoxic phase and simultaneous feeding not enhance the TN removal and even worsened the ammonia oxidation.

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# Chapter 6:

# Anaerobic digestion of aerobic granular biomass in batch experiments [1]


## Summary

The aerobic granular systems represent a good alternative to the Conventional Activated Sludge (CAS) process to reduce the amount of sludge generated in Wastewater Treatment Plants (WWTPs). However, although the quantity of sludge produced is smaller its post-treatment is still necessary. For this purpose the anaerobic digestion is widely applied in WWTPs, for the treatment of these solids. Nevertheless, up to date no information is available regarding the anaerobic digestion of aerobic granular biomass.

In the present chapter the anaerobic digestion, of raw and pre-treated by a thermal method Aerobic Granular Sludge (AGS), was studied. Two different AGS samples were tested: one collected from a reactor fed with pig manure (AGS1) and another from a reactor fed with a synthetic medium to simulate an urban wastewater (AGS2). The results obtained with the untreated AGS samples showed that their anaerobic BioDegradability (BD) (33% for AGS1 and 49% for AGS2) was similar to that obtained for a Waste Activated Sludge (WAS) (30-50%) and demonstrate the feasibility of their anaerobic digestion. The thermal pre-treatment of the biomass before the anaerobic digestion is proposed as an option to enhance the BD when this is initially low (33% in case of AGS1). This pre-treatment allowed achieving an enhancement between 20% at 60 ºC and 88% at 170 ºC with respect to the untreated sludge. However when the initial BD was high (49% in case of AGS2) the thermal pre-treatment at temperatures as high as 190-210 ºC only enhanced a little the BD (14-18%), which probably does not justify the application of such a pre-treatment.


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## 6.1. INTRODUCTION

The sewage sludge production has increased in the European Union (EU) from 5.5 million tons of dry matter in 1992 to 10.1 million tons in 2008, and it is estimated that it will reach 13.0 million tons in 2020 (http://ec.europa.eu/environment, 2011). According to the European Commission (EC) this increase is mainly due to the implementation of the Directive 91/271/EEC for Urban Waste Water Treatment (CEC, 1991) as well as the rise up in the number of households connected to sewers and in the level of required treatment. The disposal of this excess sludge represents up to 50% of the total operational costs of a Wastewater Treatment Plant (WWTP) (Appels *et al.*, 2008). The quantity of this waste which is spread on land for agricultural use is near 40% of the total produced amount in the EU and it is regulated by the Directive 86/278/EEC (CEC, 1986). In this context the sewage sludge production represents an important environmental and economic point to be considered in the design of the WWTPs and new technologies have to be developed in order to, firstly, reduce its production in the process of origin and, also, to improve its subsequent treatment.

The reduction of the sludge production in the origin is an interesting alternative. In this sense, the application of the aerobic granular technology in the WWTPs instead of the Conventional Activated Sludge (CAS) process could decrease the amount of sludge generated during the secondary treatment (Campos *et al.*, 2009). This technology, where the biomass is forced to grow in compact aggregates in Sequencing Batch Reactors (SBRs), presents various advantages in comparison with the CAS process: better settling properties and biomass retention, possibility to carry out simultaneous biological removal processes, capacity to treat higher loads, less surface requirements for its implantation and less sludge production. Liu *et al.* (2005) estimated the theoretical biomass yield of aerobic granules as 0.2 g VSS/g $COD_{removed}$, which is in accordance with the results obtained by Mosquera-Corral *et al.* (2005) operating an aerobic granular reactor fed with a synthetic medium containing acetate. This value involves a reduction of sludge production around 30% with respect to the CAS process characterized by a sludge biomass yield of around 0.3 g VSS/g $COD_{removed}$ (Heijnen and van Dijken, 1992).

However the development of the aerobic granular biomass is still recent and the research has mainly been focused on the establishment of the different optimum parameters for the reactor operation and formation of aerobic aggregates and, nowadays, on the scale up from laboratory to pilot reactors and to full scale plants. Up to now, no study has been focused on the treatment of this type of sludge before its disposal. Although Aerobic Granular Sludge (AGS) is expected to have an anaerobic degradation potential similar to the Waste Activated Sludge (WAS) due to their similar origin, specific studies are necessary to prove it.

In general the type of treatment applied to the excess sludge depends on its composition and its final application but normally the first one is a thickening step to remove the major quantity of water from the solids and reduce its volume. In this sense the thickening cost of the AGS could be lower than that of WAS due to its higher hydrophobicity (Wang *et al.*, 2005) and better settling properties (Beun *et al.*, 2000).

After thickening, a stabilization step (anaerobic digestion, aerobic digestion or composting) is commonly used to transform the amount of highly degradable organic matter into a stable waste and to reduce the number of disease-causing microorganisms present in the solids before their disposal, for example as fertilizer in agriculture. Among the biological sludge treatments the anaerobic digestion is the most suitable option due to the fact that it allows the stabilization of the sludge and also the production of energy as biogas. From previous research works it has been observed that the anaerobic biodegradability of the sewage sludge ranges from 30 to 50% depending on the type of degraded sludge and its initial organic composition (Mottet *et al.*, 2010). To improve this conversion yield many studies have been performed applying different kinds of pre-treatment (thermal, mechanical and chemical) before the sludge anaerobic digestion (Appels *et al.*, 2008; Carrère *et al.*, 2010). The main objective of these pre-treatments is to improve the solids hydrolysis rate since it is the limiting step in the anaerobic digestion and also to allow reducing the final amount of sludge to be disposed.

Carrère *et al.* (2010) compared different pre-treatment methods used to favour the biodegradability of the sludge. Although extracting a simplified conclusion is difficult, these authors observed that the low energy consuming methods, such as sonication and mechanical pre-treatment, increase the hydrolysis rate but with a limited improvement on Volatile Solids (VS) reduction, while the high energy consuming methods, such as thermal hydrolysis and oxidation, have a significant improvement on both aspects.

Although the thermal pre-treatment presents high energy consumption, the main part of the energy applied can be recovered in the form of the produced biogas during the anaerobic process (Perez-Elvira *et al.*, 2008). The literature shows that the thermal pre-treatment can be applied at different ranges of temperature and along different times of duration. Zheng *et al.* (1998) used a rapid thermal conditioning (30 s) at high temperature (220 ºC) and obtained a VS reduction of 55% and a total increase in gas production of 80%, while Gavala *et al.* (2003) applied the pre-treatment of sludge at 70 ºC during 7 days and obtained an increase of 26% in the methane production. Furthermore to know the impact that each pre-treatment has, on each sludge biodegradability, an anaerobic test under batch or continuous conditions is normally performed, which implies long operational periods (between 20 and 30 days for batch tests). In this context Mottet *et al.* (2010) proposed an estimating model to predict the anaerobic BioDegradability (BD) of WAS based on a correlation between the initial composition of the sludge and its BioMethane Potential (BMP). These authors used the partial least square (PLS) regression technique to obtain a model where both macroscopic (soluble organic carbon and COD/TOC ratio) and biochemical (carbohydrates, proteins and lipids concentrations) parameters were used to predict the anaerobic BD of WAS.

## 6.2. OBJECTIVE

The aim of this study is to test the effect of the thermal pre-treatment on the macroscopic and biochemical characteristics of the AGS and also to determine the anaerobic BD enhancement when this pre-treatment is applied. The biochemical characterization of the

samples and the results obtained with the BMP tests were also used to validate the model proposed by Mottet *et al.* (2010) to estimate the anaerobic BD of the aerobic granular biomass.

## 6.3. MATERIALS AND METHODS

### 6.3.1. Aerobic Granular Sludge (AGS) samples

The AGS samples were taken from two SBRs at pilot scale in operation with a useful volume of 100 L each. The first tested sludge (AGS1) was collected from a reactor located at the University of Santiago de Compostela (Spain) fed with the liquid fraction of pig slurry and corresponding with the Pilot Plant of Chapter 5. In this reactor the removal of organic matter and nitrogen occurred in a SBR operated in cycles of 3 hours distributed according to the following periods: 7 minutes of feeding, 168 minutes of aeration, 3 minutes of settling and 2 minutes of effluent withdrawal. The system operated at a Solid Retention Time (SRT) between 4 and 17 days. The second tested sludge (AGS2) was collected from a reactor located at the University Autònoma de Barcelona (Spain) fed with a synthetic medium which simulated an urban wastewater. In this case the removal of organic matter, nitrogen and phosphorus took place in a SBR operated with a cycle comprising anoxic and aerobic reaction phases with a cycle length of 3 hours distributed in: 60 minutes of feeding, 111 minutes of aeration, 6 minutes of settling and 3 minutes of effluent withdrawal. In this case the achieved SRT was of 20 to 40 days.

In both cases the AGS samples were taken from the solids purge stream along the reactor operational time and stored at 4 ºC. In order to remove the major quantity of water and to concentrate the sludge, the samples were settled and the supernatant was discarded. The characteristics of the AGS samples used for the experiments are presented in Table 6.1.

**Table 6.1.** Characteristics of AGS samples.

| Parameter | AGS1 | | AGS2 | |
|---|---|---|---|---|
| | Total | Soluble | Total | Soluble |
| TS (g/L) | 29.6 ± 0.2 | 1.3 ± 0.5 | 106.1 ± 2.9 | 21.0 ± 0.6 |
| VS (g/L) | 27.3 ± 0.2 | 0.9 ± 0.5 | 60.1 ± 1.2 | 13.8 ± 0.2 |
| $COD_T$ (g/L) | 39.7 ± 0.2 | 1.6 ± 0.1 | 85.7 ± 3.3 | 18.8 ± 0.9 |
| Proteins (g/L) | 16.6 ± 2.7 | 0.4 ± 0.3 | 26.9 ± 3.5 | 1.3 ± 0.2 |
| Carbohydrates (g/L) | 3.6 ± 0.2 | 0.2 ± 0.1 | 6.9 ± 1.5 | 0.5 ± 0.1 |
| Lipids (g/L) | 0.050 ± 0.007 | NA | 0.013 ± 0.001 | NA |
| PHA (g/L) | 0.8 ± 0.1 | NA | 5.5 ± 0.2 | NA |
| VFA (g/L) | NA | 1.4 ± 0.2 | NA | 7.5 ± 0.7 |

NA: Not analyzed

### 6.3.2. Thermal pre-treatment

A thermal pre-treatment was applied to the sludge samples in the range of temperatures from 60 to 210 ºC for AGS1 and from 170 to 210 ºC for AGS2. When temperatures lower than

100 ºC were applied to the sample a reactor equipped with a heating/cooling system was used and the sample volume was of 0.6 L. A Zipperclave (Autoclave France) was used when the assayed temperature values were higher than 100 ºC and the sample volume was of 0.9 L. For each experiment, once the desired temperature was reached, after 30 to 60 minutes from the beginning of the experiment, the pre-treatment was maintained during 20 minutes. Sludge samples without pre-treatment were arbitrarily associated to 20 ºC.

### 6.3.3. Analytical methods

In order to determine the composition of the AGS samples several measurements were performed on the original sludge and on the sludge after thermal pre-treatment according to the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). These measurements were carried out on the total, on the particulate and on the soluble fraction of the sample. To separate and obtain the particulate and soluble fractions each sample was centrifuged at 15,000 rpm for 15 minutes at 4 ºC (Beckman JA-20).

The measurement of the solids concentration was carried out on the total sludge (Total and Volatile Solids, TS and VS) and on the particulate fraction after sludge centrifugation (Total and Volatile Suspended Solids, TSS and VSS). The soluble fraction solids concentration was calculated as the difference between the total and the suspended ones.

Chemical oxygen demand (COD), total organic carbon (TOC), proteins and carbohydrates concentrations were determined on total sludge and on supernatant (soluble fraction), the particulate fraction was deduced from the difference between both values. The total COD ($COD_T$) was determined according to the open reflux method and the soluble COD ($COD_S$) according to the closed reflux colorimetric method (APHA-AWWA-WPCF, 2005). The TOC was measured with a Shimadzu analyzer (TOC-$V_{CSN}$) where the total sample was injected with the module SSM-5000A Shimadzu and the soluble fraction with the module ASI-V Shimadzu. The proteins concentration was measured with the Lowry method (Lowry *et al.*, 1951) and expressed in equivalent of Bovine Serum Albumin (BSA). The carbohydrates concentration was measured with the anthrone method (Dreywood, 1946) and expressed in equivalent of glucose (Glu).

Lipids concentration was measured on total sludge by accelerated solvent extraction (ASE 200, Dionex) using petroleum ether and Volatile Fatty Acids (VFA) concentration was measured in the soluble fraction by gas chromatography (GC 3900, VARIAN).

The Poly-Hydroxy-Alkanoates (PHA) concentration was measured according to a modification of the methodology used by Pijuan *et al.* (2005) and described in Chapter 2.

The average diameter of the granules in the samples was determined by using an Image Analysis procedure (Tijhuis *et al.*, 1994) with a stereomicroscope (Stemi 2000-C, Zeiss) for particles with a size higher than 1 mm and by using a laser radiation technique (Beckman Coulter LS200 equipped with a LS Variable Speed Fluid Module Plus) for particles with a size lower than 1 mm.

### 6.3.4. Anaerobic biodegradability tests

The tests were carried out in glass flaks (Figure 6.1) at 35 ºC and as was described in Chapter 2. From each sample three different fractions were analysed: a) the liquid fraction of sludge; b) the solid fraction of sludge and c) the whole sludge. The first two determinations were performed to know the contribution of each phase to the biodegradability of the sample while the third one was done to check the mass balance.

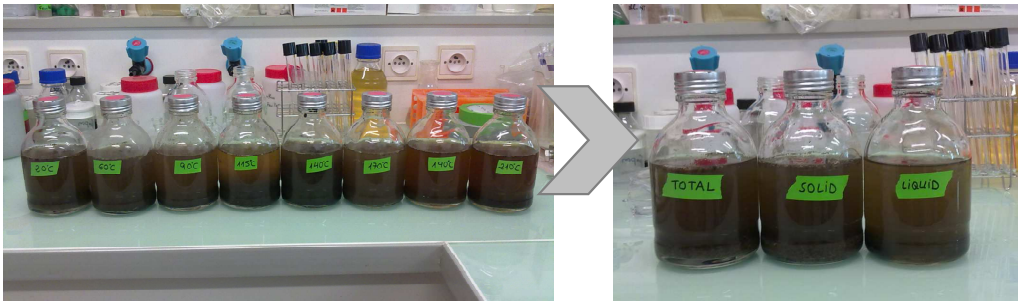


**Figure 6.1.** Images of glass flaks for AGS1 samples at different temperatures of pre-treatment and for each sample fraction.

### 6.3.5. Calculations

***BioMethane Potential (BMP) and BioDegradability (BD)***

The BMP obtained with the batch anaerobic tests was expressed as the volume of methane produced per gram of total COD of substrate fed in standard conditions (N-mL $CH_4$/g $COD_{fed}$). The BD was determined according to Mottet *et al.* (2010) by dividing the BMP by the maximum theoretical $CH_4$ production in standard conditions (350 N-mL $CH_4$/g $COD_{fed}$ at 1 atm and 0 °C).

The estimation of the BD percentage of the sludge ($BD_{model}$) was calculated using the equation [6.1] according to the model proposed by Mottet *et al.* (2010). This model correlates the BD and the sludge composition to predict its anaerobic BD in terms of: proteins (Prot; g BSA/g VS), carbohydrates (Carb; g Glu/g VS), lipids (Lpd; g/g VS), COD/TOC ratio (Ox; g COD/g TOC) and soluble organic carbon (SolOc; g TOC/g VSS). The error associated to the model respect to the values measured with the batch anaerobic tests was calculated using the equation [6.2].

$$BD_{model} = (0.043 - 0.106 \cdot Prot + 0.661 \cdot Carb + 0.836 \cdot Lpd + 0.074 \cdot Ox + 0.349 \cdot SolOc) \cdot 100 \quad [6.1]$$

$$Error = \frac{|BD_{model} - BD|}{BD} \cdot 100 \quad [6.2]$$

***Specific coefficient rates***

The specific coefficient rate corresponding to the particulate fraction ($k_P$; $d^{-1}$) was calculated based on a first order reaction kinetic (Redzwan and Banks, 2004) and according to the equation [6.3], where "$X_P$" is the substrate/inoculum ratio (g COD/g $VS_{inoculum}$), correlated to the biodegradable particulate fraction of COD and obtained by the cumulative methane production curve. To determine the value of $k_P$ a linear form of this equation was derived (equation [6.4]), where: "$X_P^0$" is the biodegradable substrate/inoculum ratio at the beginning of the experiment (g COD/g $VS_{inoculum}$), "$X_P^t$" is the biodegradable substrate/inoculum ratio at certain moment (g COD/g $VS_{inoculum}$) and "t" is the digestion time (d) for the particulate fraction.

$$-\frac{dX_P}{dt}=k_P \cdot X_P \qquad [6.3]$$

$$\ln\left(\frac{X_P^0}{X_P^0-X_P^t}\right)=k_P \cdot t \qquad [6.4]$$

The Monod model (equation [6.5]) was used to describe the consumption rate of the soluble COD ($r_S$), where "$r_{S-max}$" is the maximum rate for the soluble phase (g COD/g $VS_{inoculum}\cdot d$), "$k_S$" is the half saturation constant (g COD/L) and "$S_S$" the initial concentration of substrate in the liquid media correlated to the biodegradable soluble fraction of COD (g COD/L). To determine the parameters $k_S$ and $r_{S-max}$ a linear form of the Monod equation according to Lineweaver-Burk model was used (equation [6.6]). For each experiment $r_S$ was calculated based on the initial rate method as the initial slope of the cumulative curve of methane production for the soluble fraction, expressed as COD and referred to the amount of inoculum (g COD/g $VS_{inoculum}\cdot d$).

$$r_S=r_{S-max}\cdot\frac{S_S}{k_S+S_S} \qquad [6.5]$$

$$\frac{1}{r_S}=\frac{1}{r_{S-max}}+\frac{k_S}{r_{S-max}}\cdot\frac{1}{S_S} \qquad [6.6]$$

## 6.4. RESULTS AND DISCUSSION

### 6.4.1. Effects of thermal pre-treatment on AGS characteristics

The variation of the $COD_T$, proteins, lipids and carbohydrates contents of sample AGS1 after being submitted to the different tested temperatures was evaluated (Figure 6.2). The $COD_T$, proteins and lipids content remained almost constant for all the assayed temperatures, which mean that they were not degraded by the heat. The decrease of carbohydrates content could be attributed to the own measurement method. As Bougrier *et al.* (2008) explained the carbohydrates may react with other carbohydrates ("burnt sugar" reactions), which provokes the disappearance of the carbonyl group (C=0), that is used in their quantification. This is the reason of their underestimation. The results for sample AGS2 are similar to those exposed for AGS1.

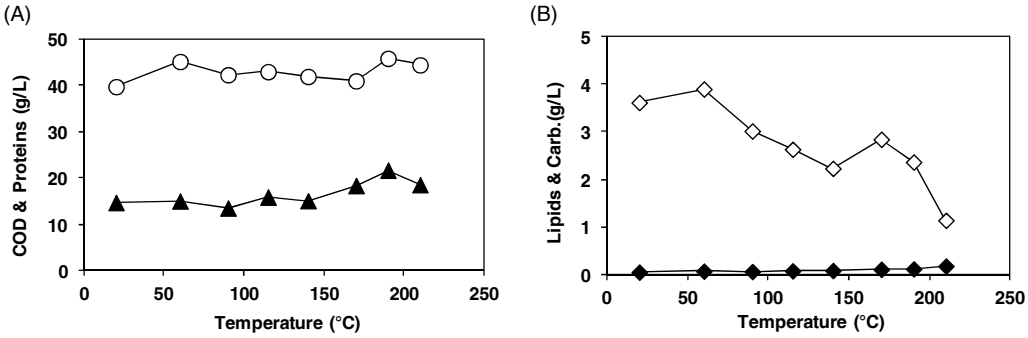


**Figure 6.2.** Concentrations of the different compounds on the sample AGS1: (A) $COD_T$ (○) and proteins (▲); (B) lipids (◆) and carbohydrates (◇).

The measured concentration of lipids was low, between 0.05-0.18 g/L for AGS1 and 0.01-0.10 g/L for AGS2, in comparison with the concentrations observed in activated sludge samples, between 0.24 and 3.4 g/L (Mottet *et al.*, 2010). This is probably due to the low fats content in the feeding of the reactors of origin (pig manure and synthetic urban wastewater) compared with an urban wastewater. The VFA concentration in the soluble phase remained constant around 1.4 g/L and 7.5 g/L for AGS1 and AGS2, respectively (data not shown).

In sample AGS1 (from the reactor treating pig manure to remove C and N) the organic fraction represented 92% of the total solids, while in sample AGS2 (from the reactor treating synthetic urban wastewater to remove C, N and P) this fraction only represented 55-60% (Figure 6.3). The high proportion of the mineral fraction in the sludge AGS2 can be associated to the removal of phosphorus and the presence of a precipitate material in the core of some granules (Figure 6.4). De Kreuk *et al.* (2005) operated a SBR with aerobic granular biomass and observed that the mineral content of the granules increased from 6% to 30-41% when the removal of phosphorus occurred. These authors explained that part of the phosphate removal might be caused by the precipitation of apatite inside the granules.

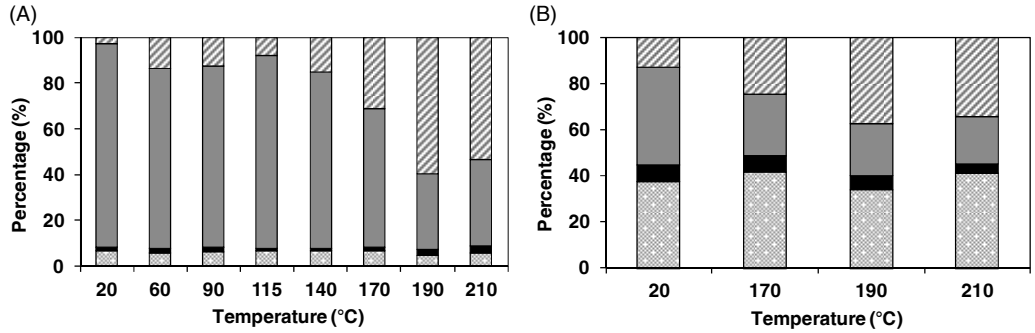


**Figure 6.3.** Solids repartition between the particulate and soluble fractions: (A) AGS1 and (B) AGS2. Particulate mineral fraction (▨), soluble mineral fraction (■), particulate organic fraction (■) and soluble organic fraction (▨).

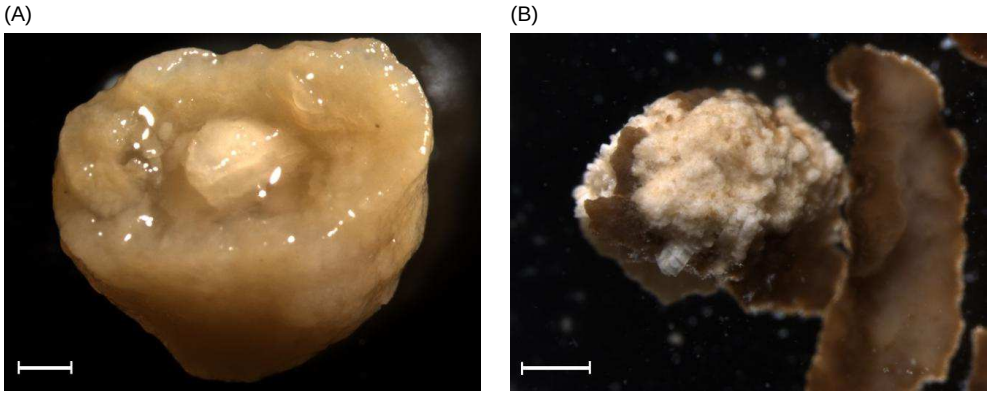


**Figure 6.4.** (A) Image of the core of a granule of AGS2; (B) image of the precipitate with rests of biomass after the thermal pre-treatment at 170 ºC. The size bar is 0.5 mm.

The main effect of the thermal pre-treatment on both sludge samples was the solubilization of the organic fraction, while the mineral fraction was not affected (Figure 6.3). For the experiments performed with temperatures below 115 ºC the percentages of solubilization obtained for AGS1 samples were similar and they seemed to be independent from the temperature (Figure 6.5). However in the case of temperatures above this value the percentages of solubilization increased quickly with the rise of the temperature. To explain this behaviour it has to be taken into account that in the experiments of thermal pre-treatment at 60 ºC and 90 ºC the AGS1 presented a viscous aspect similar to that of a gel, reaching the maximum gel compactness structure at 115 ºC. After removing the sample from the interior of the thermal reactor a "block" of sludge was obtained (Figure 6.6.A). This behaviour is the opposite to the tendency observed for activated sludge by Bougrier *et al.* (2008). These authors measured the apparent viscosity of WAS after the thermal pre-treatment (temperatures between 20 ºC and 190 ºC) and they observed that it decreased with the increase of the pre-treatment temperature. An explanation for this gelatinous aspect at temperatures below or equal to 115 ºC could be the high Extracellular Polymeric Substances (EPS) content, with gel-forming properties, of the aerobic granular biomass with respect to the activated sludge (Seviour *et al.*, 2009). Indeed, at these moderate temperatures the EPS were slightly released from the surface of the granules to the media and they acted as a bond to maintain the gel structure. However, at high temperatures the EPS solubilization was higher, as it can be observed on Figure 6.5 (high percentage of proteins and carbohydrates solubilization), probably due to the loss of their gel-forming properties.

The maximum solubilization values were obtained at temperature of 190 ºC for all the parameters measured. For higher temperatures the solubilisation percentage of proteins remained almost constant while a decrease of 30% for carbohydrates, 14% for VS and 4% for COD was measured. In case of sample AGS2 the results obtained with the studied temperatures (170, 190 and 210 ºC) were similar to those obtained from sample AGS1:

maximum solubilization at 190 ºC (except for the carbohydrates with a maximum solubilization value of 42% at 170 ºC) and then a decrease at 210 ºC. Bougrier *et al.* (2008) also observed the maximum solubilization percentages around 170 to 190 ºC for activated sludge, while for higher temperatures the tendency was not clear. This fact could be attributed to the occurrence of reactions between an amino acid and a carbohydrate (Maillard reaction), usually requiring heat, to form complex molecules. The occurrence of this type of reactions with high temperatures could also be visually appreciated with the colour change to dark brown of the soluble phase (Figure 6.6.B).

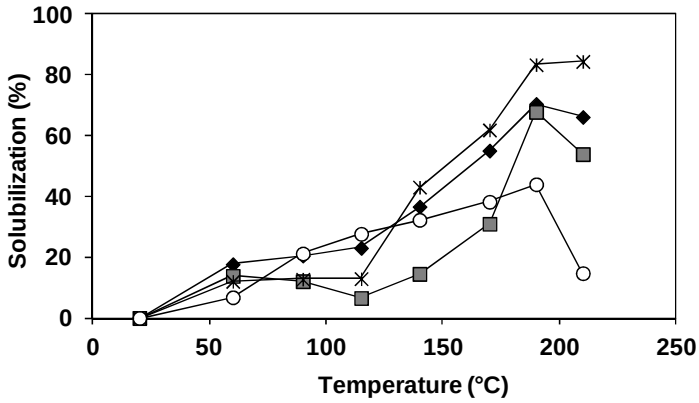


**Figure 6.5.** Solubilization percentages of COD (◆), VS (■), proteins (∗) and carbohydrates (○) for AGS1 sample after the thermal pre-treatment.

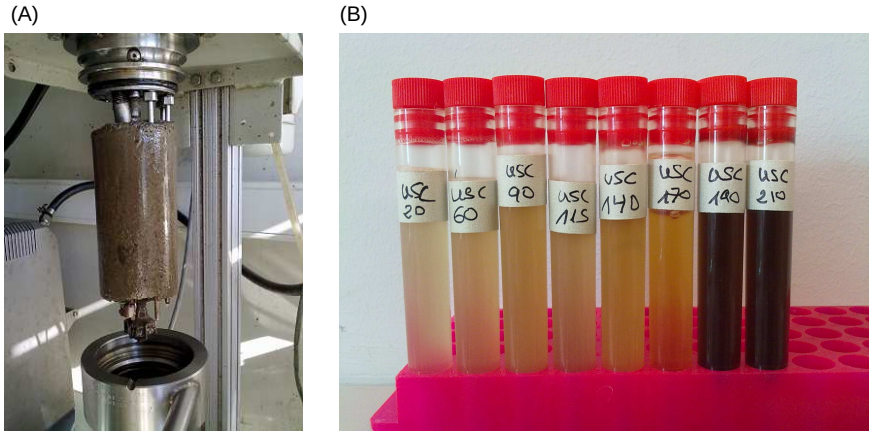


**Figure 6.6.** (A) Image of the aerobic granular biomass AGS1 after the thermal pre-treatment at 115 ºC and (B) image of the soluble phase at different temperatures of pre-treatment for AGS1.

The solubilization percentages obtained for each parameter with both AGS samples and those from WAS at 190 ºC are shown on Table 6.2. For all the parameters measured, the

percentage of solubilization was higher in sample AGS1 than in sample AGS2. The solubilization values obtained for aerobic granules (AGS1 and AGS2) were in the same range as those reported for the activated sludge samples, except for the proteins, which had a higher solubilization percentage in the case of both granular sludge.

**Table 6.2.** Comparison of the solubilization percentages at 190 ºC of samples AGS1, AGS2 and several WAS samples.

| Sample | $S_{VS}$ (%) | $S_{COD}$ (%) | $S_{Proteins}$ (%) | $S_{Carbohydrates}$ (%) |
|---|---|---|---|---|
| AGS1 | 64 | 70 | 83 | 44 |
| AGS2 | 35 | 57 | 76 | 34 |
| WAS* | 40-80 | 50-82 | 32-60 | 16-45 |

*WAS: values corresponding to five activated sludge (Bougrier *et al.*, 2008).

The thermal pre-treatment also provoked changes on the pH of the liquid media and on the average diameter of the granules (Table 6.3). The decrease of pH with the increase of temperature could be associated to the formation of acid compounds due to the degradation of macromolecules. However the production of VFA was not detected. The average diameter of the aerobic granules moderately decreased with the temperatures between 60 and 115 ºC and it strongly decreased with temperatures higher than these ones (Laurent *et al.*, 2009). However for temperatures between 140 and 210 ºC it was impossible to obtain a tendency on the evolution of the average diameter, probably due to the error associated to the measurement methodology. Then it is possible to conclude that, as it was mentioned before, at low temperatures the gel-forming structure is responsible for the low diameter reduction, while when the temperature is above 115 ºC the reduction on the average diameter is clearly observed. The determination of the average diameter at 115 ºC was not possible due to the gel structure of the sample.

**Table 6.3.** Values of pH and particle average diameter after pre-treatment at different temperatures.

| Temperature (ºC) | | 20 | 60 | 90 | 115 | 140 | 170 | 190 | 210 |
|---|---|---|---|---|---|---|---|---|---|
| pH | AGS1 | 7.11 | 7.29 | 7.17 | 7.07 | 6.97 | 6.4 | 6.29 | 6.24 |
| | AGS2 | 6.50 | - | - | - | - | 5.48 | 5.31 | 5.16 |
| Diameter (μm) | AGS1 | 1630 | 1380 | 1080 | ND | 175 | 181 | 106 | 140 |
| | AGS2 | 2610 | - | - | - | - | 118 | 155 | 120 |

ND: Not determined

### 6.4.2. Anaerobic biodegradability of AGS

To evaluate the effect of the thermal pre-treatment on the anaerobic BD of the AGS batch tests were performed after the application of the assayed temperature to the biomass samples. The cumulative methane production per gram of COD fed for the different pre-treatment conditions was followed in duplicate assays (Figure 6.7). The methane production from the control experiments, performed without substrate addition, was subtracted from the previous obtained value. The percentage of BD for each sample is indicated in Table 6.4.

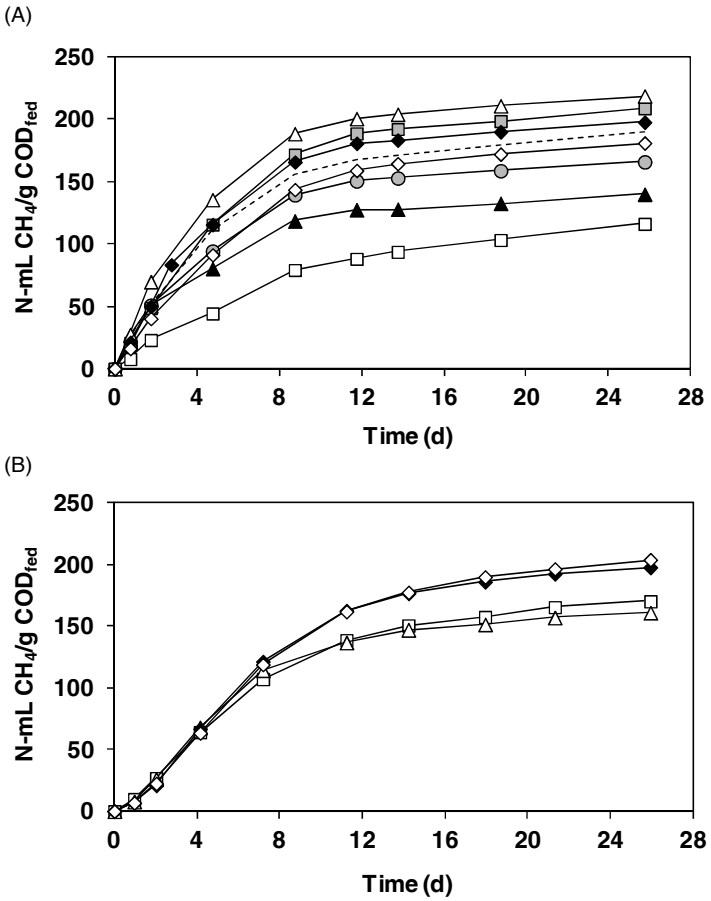


**Figure 6.7.** Average cumulative methane production during the batch anaerobic digestion tests: (A) sample AGS1 and (B) sample AGS2. Temperature of pre-treatment: 20 ºC (□), 60 ºC (▲), 90 ºC (●), 115 ºC (---), 140 ºC (■), 170 ºC (△), 190 ºC (◆) and 210 ºC (◇).

The BD of the sample AGS1 without pre-treatment (20 ºC) was around 33% and although it is in the range of the BD for WAS (30-50%), this value was near the low limit and indicated a poor BD of AGS1. The thermal pre-treatment caused an improvement of the anaerobic BD for all the temperatures tested with the sample AGS1. The BD increased until a maximum value of 62% at 170 ºC, which corresponds to an enhancement of the BD of 88% with respect to the

original sample. Then pre-treatments at higher temperatures decreased the BD, but it remained higher than that of the untreated sludge.

The BD of the sample AGS2 without pre-treatment (20 ºC) was of 49% and it was not improved by the thermal pre-treatment at 170 ºC. Only when the tested temperatures were as high as 190 and 210 ºC the BD increased slightly up to 56 and 58%, respectively, which supposed an enhancement between 14 and 18% with respect to the untreated sample AGS2. The necessity to apply high temperatures to obtain only a little improvement in the BD for sludge AGS2, would not justify the application of a thermal pre-treatment for this sludge. But it is still interesting to note that for this sludge at temperatures over 190 ºC the tendency of the BD was to increase, whereas the contrary was observed with sample AGS1 and with sewage sludge (Pinnekamp, 1989; Bougrier *et al.*, 2008).

Although sample AGS1 without pre-treatment had a smaller granule average diameter and a lower sludge age (SRT on the reactor of origin) than AGS2, which could favor the BD, the opposite was observed. The different results obtained with both aerobic granular samples could be explained by their different concentration of PHA and VFA (easily biodegradable compounds). The concentration of these compounds represented the 3.4% and 4.7% of the $COD_T$ for PHA and VFA, respectively, in case of sample AGS1. However for sample AGS2 the percentages of PHA and VFA represented a higher contribution with 11.5% and 12.8% of the $COD_T$, respectively. If it is considered that the PHAs and VFAs were completely degraded during the anaerobic digestion, the contribution of these compounds to the biodegradability represented the 8.1% for AGS1 and the 24.3% for AGS2, being the difference of 16.2%, a similar value to that observed in the BMP tests.

**Table 6.4.** Results of the anaerobic BD tests with samples AGS1 and AGS2 after thermal pre-treatment.

| Sample | Temperature (ºC) | BMP (N-mL $CH_4$/g $COD_{fed}$) | BD (%) | $BD_{model}$ (error) |
|---|---|---|---|---|
| AGS1 | 20 | 116 ± 5 | 33 ± 1 | 32 (2%) |
| | 60 | 140 ± 7 | 40 ± 2 | 44 (11%) |
| | 90 | 166 ± 4 | 47 ± 1 | 41 (13%) |
| | 115 | 190 ± 8 | 54 ± 2 | 55 (1%) |
| | 140 | 209 ± 10 | 60 ± 3 | 52 (12%) |
| | 170 | 219 ± 4 | 62 ± 1 | 55 (12%) |
| | 190 | 198 ± 3 | 56 ± 1 | 46 (18%) |
| | 210 | 181 ± 10 | 52 ± 3 | 44 (15%) |
| AGS2 | 20 | 170 ± 1 | 49 ± 0 | 41 (16%) |
| | 170 | 161 ± 3 | 46 ± 1 | 47 (3%) |
| | 190 | 198 ± 8 | 56 ± 2 | 53 (6%) |
| | 210 | 204 ± 11 | 58 ± 3 | 53 (8%) |

The differences between the BD of both AGS samples were also observed with the thermal pre-treatment (Table 6.4). In the case of AGS1 the temperature of 170 ºC was the optimal one for the BD enhancement, while it did not induce any change for AGS2. At 190 and 210 ºC the values of BD obtained with both samples were similar; however the enhancement with respect to the original BD was higher in AGS1. This is in accordance with Bougrier *et al.* (2008) who observed that the BD enhancement was larger with lower initial BD and higher COD solubilization after the thermal pre-treatment. In this study similar results were observed indicating that sample AGS1, which had the lower initial BD, presented also the higher solubilisation percentage after the thermal pre-treatment in comparison with sample AGS2 (Table 6.2). The different substrate used to fed the reactors where the samples were taken from (pig manure for AGS1 and synthetic medium for AGS2) could be the responsible for the different characteristics of the aerobic granules, being their compositions determinant in influencing the potential anaerobic biodegradability.

Recently Mottet *et al.* (2010) developed a model based on empirical results that correlates the BD and the sludge composition to predict the anaerobic BD of activated sludge. This model was used to estimate the $BD_{model}$ at different pre-treatment conditions assayed for the granular sludges, AGS1 and AGS2, used in this work (Table 6.4). The values obtained from the model are quite similar to those experimentally obtained, the differences among them ranging from 2 to 18%. These differences are “acceptable” taking into account that the prediction error obtained by the authors for activated sludge was of 11%. So this model seems to be adequate to predict the anaerobic BD of both, activated or granular sludge.

The enhancement of the anaerobic BD with the thermal pre-treatment was associated to the solubilisation of the different compounds. On Figure 6.8 the relation between the BD enhancement respect to the untreated sludge and the COD solubilisation is presented for both granular sludges. In the case of sample AGS1 a relation was observed for values of COD solubilisation lower than 60% (temperature of pre-treatment lower than 190 ºC). However for sample AGS2 although the COD solubilisation percentage was between 30 and 60% the BD enhancement was very low.

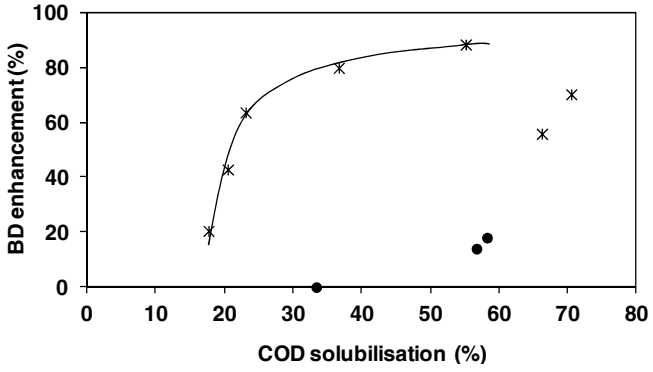


**Figure 6.8.** Relation between BD enhancement and COD solubilisation for AGS1 (⁎) and AGS2 (●).

### 6.4.3. Particulate and soluble fractions contribution to the biogas production

Independent batch assays were carried out with soluble and particulate fractions of both sludge samples to determine their contribution to the methane production (Figure 6.9 and Figure 6.10 for AGS1 and AGS2, respectively).

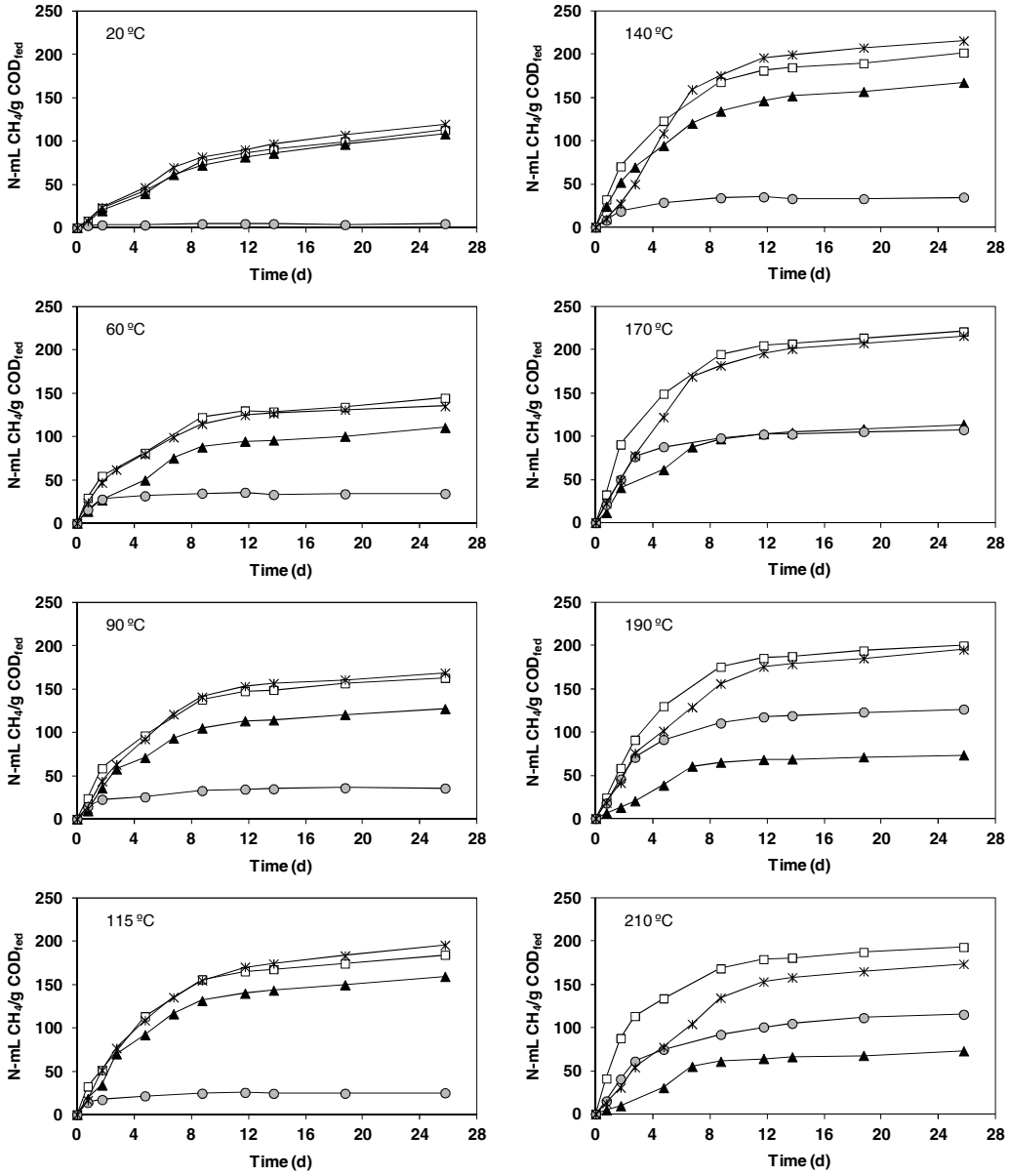


**Figure 6.9.** Cumulative methane production during the batch anaerobic tests of sludge AGS1 at different temperatures of pre-treatment for the total sample (∗), the particulate fraction (▲), the soluble fraction (●) and the sum of particulate and soluble fractions (□).

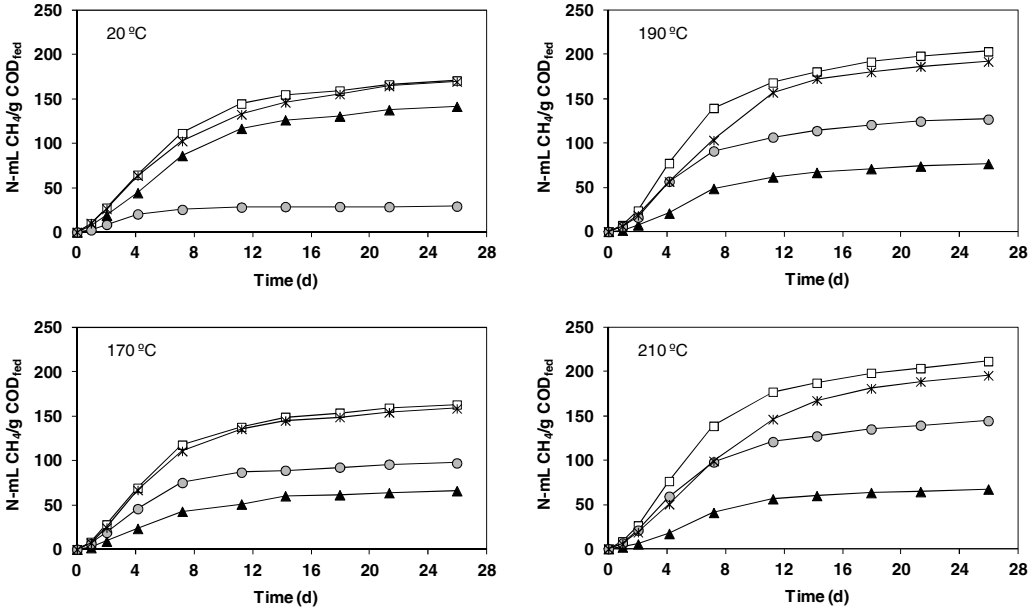


**Figure 6.10.** Cumulative methane production during the batch anaerobic digestion tests of sludge AGS2 at different temperatures of pre-treatment for the total sample (∗), the particulate fraction (▲), the soluble fraction (●) and the sum of particulate and soluble fractions (□).

The methane production by each fraction during the batch tests was taken into account and the obtained total amount compared to that obtained in batch tests done with the whole sludge samples. The good obtained fits indicate that the mass balances matched well.

In the case of sample AGS1 without pre-treatment the methane production was mainly due to the particulate fraction (96%) and the contribution of the soluble phase increased from 4% at 20 ºC to 17% at 140 ºC (Figure 6.9). At 170 ºC the contribution of both phases to the methane production was similar and up to this value the contribution of the soluble phase was higher (62%) than that of the particulate one (38%).

For sample AGS2 without pre-treatment the methane production was also mainly due to the particulate fraction (83%), although for this granular sludge the contribution of the soluble fraction without pre-treatment (17%) was higher than for AGS1 (Figure 6.10). The methane production for both fractions at the high temperatures of pre-treatment tested with this sludge (170-210 ºC) was similar to that corresponding to AGS1 samples.

The contribution of the soluble and particulate fractions to the total methane production with the thermal pre-treatment was influenced not only by the solubilisation of the compounds but also by the change on the BD of each fraction, as is shown on Figure 6.11.

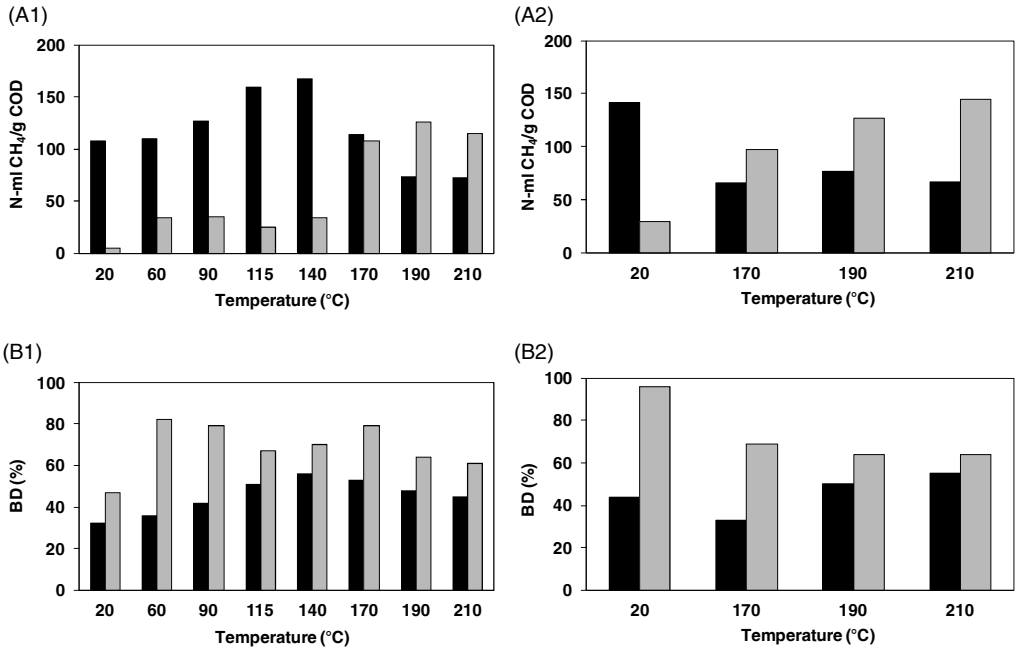


**Figure 6.11.** (A) Methane volume produced at the end of the batch anaerobic digestion for particulate (■) and soluble (■) fractions per gram of COD on the total sample. (B) BD percentage of particulate (■) and soluble (■) fractions. (1) AGS1 and (2) AGS2 samples.

For sample AGS1 the contribution of the particulate phase to the total methane production increased at temperatures between 60 and 140 ºC, despite that the solubilisation caused a decrease on its organic matter content. This was due to the fact that the particulate phase became more biodegradable with the thermal pre-treatment on this range of temperatures. When values higher than 170 ºC were applied the BD of the particulate and soluble phase started to decrease indicating that with high temperatures both phases became more recalcitrant. The maximum contribution of the particulate phase was obtained at 140 ºC that coincides with its higher BD, while the maximum contribution of the soluble phase was at 190 ºC that coincides with the higher solubilisation percentage. The combination of both effects causes that for AGS1 the higher values of BMP and BD were obtained for the pre-treatment at 170 ºC (Table 6.4).

For sample AGS2 the BD of the particulate fraction pre-treated at 170 ºC was the lowest one from all the tested temperatures and even lower than that of the sample without pre-treatment (Figure 6.11.B), which could explain the low BD obtained with a pre-treatment at 170 ºC for AGS2 in comparison with sample AGS1 and WAS. The contribution of the particulate fraction pre-treated at 190 and 210 ºC was in the same range as AGS1 (70-75 N-ml $CH_4$/g $COD_{fed}$). With the increase in the pre-treatment temperature the BD of the soluble fraction of

AGS2 decreased (became more recalcitrant) and the BD of the particulate fraction increased (became more available).

The rate of the process for both fractions degradation was also analysed in order to know how the thermal pre-treatment influences it. Degradation of the particulate fraction fits to the first order model proposed by Redzwan and Banks (2004) (equation [6.4]). The values of $k_P$ obtained (Table 6.5) ranged between 0.119-0.187 $d^{-1}$ and 0.159-0.174 $d^{-1}$ for samples AGS1 and AGS2, respectively, and matched the literature values (Pavlostathis and Giraldogomez, 1991; Batstone *et al.*, 2009; Donoso-Bravo *et al.*, 2010). The higher the $k_P$ value the faster the degradation of the particulate fraction, so comparing the values obtained for both AGS samples without pre-treatment (20 ºC) the degradation of the particulate fraction of AGS2 was faster than for AGS1. The thermal pre-treatment increased the values of $k_P$ respect to the untreated samples in all the cases (except at 210 ºC for sample AGS2), although this increase was slight in the case of AGS2. The higher values of $k_P$ were obtained at 170 ºC for both AGS samples.

**Table 6.5.** Values of $k_P$ and correlation coefficient $R^2$ of particulate fraction at different pre-treatment temperatures for AGS1 and AGS2 samples.

| **Temperature (ºC)** | | **20** | **60** | **90** | **115** | **140** | **170** | **190** | **210** |
|---|---|---|---|---|---|---|---|---|---|
| **AGS1** | **$k_P$ ($d^{-1}$)** | 0.119 | 0.166 | 0.178 | 0.159 | 0.174 | 0.187 | 0.184 | 0.169 |
| | **$R^2$** | (0.996) | (0.966) | (0.983) | (0.981) | (0.980) | (0.983) | (0.985) | (0.993) |
| **AGS2** | **$k_P$ ($d^{-1}$)** | 0.164 | - | - | - | - | 0.174 | 0.172 | 0.159 |
| | **$R^2$** | (0.990) | | | | | (0.995) | (0.994) | (0.994) |

Experimental results show that the consumption rate of the biodegradable soluble fraction of COD presented a good data correlation with the Monod model (Table 6.6). The $k_S$ values obtained in this work are in the range of those obtained by other authors for the anaerobic digestion of long chain fatty acids (1.27–4.62 g COD/L) (Pavlostathis and Giraldogomez, 1991; Jeyaseelan, 1997) and higher than those obtained for carbohydrates (0.45 g COD/L), proteins (0.50 g COD/L) or lipids (0.85 g COD/L) (Jeyaseelan, 1997). The values of $k_S$ and $r_{S\text{-}max}$ for both sludge samples were different probably due to their different composition.

**Table 6.6.** Kinetic parameters for soluble fraction of AGS1 and AGS2.

| **Sample** | **$k_S$ (g $COD_S$/L)** | **$r_{S\text{-}max}$ (g $COD_S$/g $VS_{inoculum}$·d)** | **$R^2$** |
|---|---|---|---|
| AGS1 | 2.803 | 0.273 | 0.995 |
| AGS2 | 1.168 | 0.068 | 0.998 |

In this study, it was observed that the anaerobic hydrolysis of the particulate biodegradable fraction adjusts to a first order kinetic while the consumption rate of the soluble biodegradable fraction follows a Monod kinetic, which coincided with the study of Massé and Droste (2000). Furthermore, since the mass balance, calculated with the results from the total sample and those obtained from the addition of results from particulate and soluble fractions, matched well, the consumption rate of the total sample ($r_T$) can be determined as the sum of the particulate and soluble COD biodegradable fractions consumption rates (Figure 6.12). The values of $r_T$ increased for sample AGS1 with the temperature applied on the thermal pre-treatment. However for sample AGS2 the pre-treatment temperatures assayed did not imply a significant change on the values of $r_T$, they were even slight lower than those of the untreated sludge. In Figure 6.12 it is shown that the total rates of the process for the untreated sludges were similar for both samples (0.08 g COD/g $VS_{inoculum}$·d).

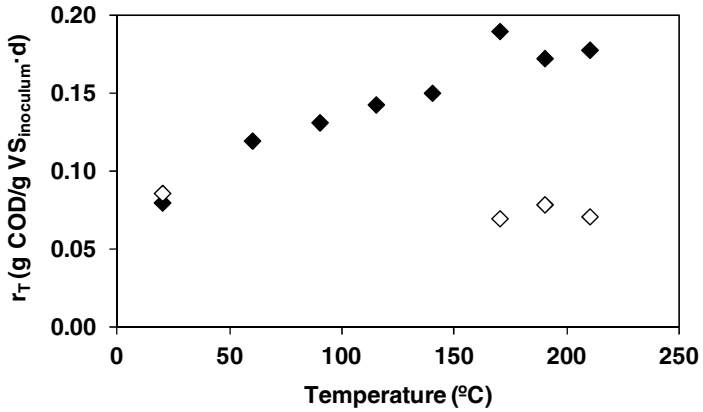


**Figure 6.12.** COD consumption rate of the total sample per gram of $VS_{inoculum}$ at different temperatures for AGS1 (◆) and AGS2 (◇) samples.

Therefore degradation kinetic was influenced by the thermal pre-treatment in such a way that the maximum BMP and BD of the AGS1 sample were obtained at 170 ºC. For the AGS2 sample the thermal pre-treatment, even at high temperatures, did not improve the COD consumption rate along the anaerobic digestion that, together with the low BD enhancement, confirms the low viability of the thermal pre-treatment of this AGS sample.

## 6.5. CONCLUSIONS

The thermal pre-treatment had a little effect on the total composition of the aerobic granular samples, but an important effect on the solubilization of the organic compounds. The maximum solubilization yield for both aerobic granular biomasses assayed was observed at 190 ºC.

The anaerobic BD of AGS samples without pre-treatment (33% and 49% for AGS1 and AGS2, respectively) was similar to that reported for WAS (30-50%) and demonstrated the feasibility of their anaerobic digestion.

In the case of the aerobic granules from a reactor treating pig manure for carbon and nitrogen removal the initial BD was of 33% and for all the temperatures tested (60-210 ºC) the pre-treatment led to an enhancement of this parameter between 20% (60 ºC) and 88% (170 ºC). Furthermore the rate of the anaerobic digestion process was improved by this thermal pre-treatment. Therefore the thermal pre-treatment was proposed to enhance the anaerobic digestion of AGS when its initial BD is low. The results obtained were similar to those from previous works with WAS.

For the aerobic granules from a reactor treating a synthetic wastewater for carbon, nitrogen and phosphorous removal the initial BD was higher (49%) than the previous one, but the thermal pre-treatment only enhanced a little the methane production of 14 and 18% at high temperatures of 190 and 210 ºC, respectively. In addition the kinetic of the anaerobic digestion did not change and even the COD consumption rate was slightly lower than without pre-treatment. These facts definitively did not justify the application of such a pre-treatment.

The results obtained in this work allowed validating the model developed on WAS by Mottet *et al.* (2010) for its application to the case of AGS. This model calculation allows the estimation of the BD of a sludge based on its chemical characteristics not on the aggregation properties of the biomass.

The consumption rate of the particulate biodegradable fraction followed a first order model while the kinetic of the soluble biodegradable fraction had a good correlation data to a Monod model. The mass balance between the BMP of total sample and the sum of the BMPs of particulate and soluble fractions matched well, so the overall rate of the process can be inferred as the sum of particulate and soluble ones.

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# Chapter 7:

# Continuous anaerobic digestion of aerobic granular biomass: effects of thermal pre-treatment and addition of primary sludge


## Summary

The aerobic granular systems are proposed as a good alternative to the Conventional Activated Sludge (CAS) ones due to the lower amount and better settleabilty properties of the produced sludge. However the generated Aerobic Granular Sludge (AGS) also needs to be treated before disposal. The AGS has a similar origin than the Waste Activated Sludge (WAS), which means that both have similar composition and that the anaerobic digestion could be a suitable treatment for this biomass as it is applied to the WAS. However the fact that the biomass is aggregated in granules can imply the presence of mass transfer limitations during the application of the anaerobic degradation.

The results obtained during the discontinuous experiments described in Chapter 6 revealed that the anaerobic digestion of AGS has a similar productivity than that obtained from the treatment of WAS in terms of methane production. Based on these previous results the feasibility of the AGS anaerobic digestion in continuous conditions is studied in the present chapter.

A continuous fed-batch anaerobic digester was operated in three different stages. Initially it was fed with raw AGS, later with thermal pre-treated AGS and to finish with a mixture of thermal pre-treated AGS with Primary Sludge (PS). The anaerobic digestion process operated in stable conditions during the operational period. The values of BioDegradability (BD) achieved in the case of the raw AGS were of 44% and the solids reduction of 32%. These values were similar to those reported for WAS. The thermal pre-treatment of the AGS at 133 ºC enhanced the reactor performance 32% and 47% in terms of BD and solids reduction, respectively. The mixture of thermal pre-treated AGS with PS provided better results of solids removal than in the case with only thermal pre-treated AGS, but lower values of BD due to the high $COD_T$/VS ratio. The application of the FISH (Fluoresecence *In Situ* Hybridization) technique to the microbial populations of the anaerobic sludge revealed that the microorganisms belonging to *Archaea* domain were more abundant than with those included in the *Bacteria* domain. The abundance of both domains decreased with the change on the feeding composition from raw AGS, to pre-treated AGS and its mixture with PS.

## 7.1. INTRODUCTION

Nowadays in the developed countries the generation of high amounts of waste and their subsequent management constitutes a great drawback. The production of wastewater, from urban or industrial origin, is an example of these problems. In this sense in the European Union the Directive 91/271/EEC for Urban Waste Water Treatment (CEC, 1991) has as main objective to protect the environment from the adverse effects of urban wastewater discharges and discharges from certain industrial sectors. To fulfil these requirements wastewater treatment systems are applied with the consequent sludge production, which as a waste needs to be treated.

In the Wastewater Treatment Plants (WWTPs) the sludge is generated mainly from the primary treatment (Primary Sludge, PS) and the secondary treatment that involves biological processes (Waste Activated Sludge, WAS). To treat these solids a stabilization step of the sludge is commonly applied to reduce the pathogens content followed by a dehydration step to decrease the volume. One of the most used technologies to stabilize the sludge is the anaerobic digestion, due to its ability to reduce the amount of sludge solids for final disposal, to destroy most of the pathogens present in the sludge, to limit possible odour problems associated to residual putrid matter and to transform organic matter into biogas which can be used to produce energy in the same installation, for example to heat the anaerobic digester or to dry the final sludge (Appels *et al.*, 2008).

The anaerobic biodegradability of the WAS is limited by the hydrolysis step (Bougrier *et al.*, 2007) and also the low C/N ratio of the sludge composition (between 6 and 16) is reported as a problem for the anaerobic digestion (Stroot *et al.*, 2001). The suggested optimum C/N ratio is in the range from 20 to 30 (Hawkes, 1980) to ensure the presence of sufficient nitrogen supply for the cell production and the biological degradation of the organic matter. The co-digestion of WAS with PS can be a solution to overcome the difficulties of treating WAS alone and to adjust its unbalanced nutrients quantity (Bouallagui *et al.*, 2010). These authors observed that the addition of PS to WAS during the anaerobic digestion is beneficial in the way that it increases the solids removal and the specific biogas production.

The anaerobic biodegradability of the PS is normally higher than that of the WAS (Bougrier *et al.*, 2006; Ekama *et al.*, 2007; Perez-Elvira *et al.*, 2008). Thereby the application of a pre-treatment before the anaerobic digestion is often used to enhance the biodegradability of the WAS in preference to PS pre-treatment (Haug *et al.*, 1983; Carrère *et al.*, 2010), because the energy needed to pre-treat the PS is too high compared to the subsequent little increase in the amount of obtained biogas (Perez-Elvira *et al.*, 2008). The pre-treatment methods to improve the anaerobic biodegradability of the sludge include biological, thermal, mechanical and chemical processes (Carrère *et al.*, 2010). Among them the thermal pre-treatment is reported as one of the most widely used methods because the energy input needed could be satisfied by the energy obtained from the biogas production enhancement in the anaerobic digester, achieving an energetically self-sufficient process (Perez-Elvira *et al.*, 2008). The conditions to apply the

thermal pre-treatment can vary and depend on the characteristics of the sludge, but most of the studies agree that the best strategy is to apply a temperature of 160-180 ºC during 30 to 60 minutes (Carrère *et al.*, 2010).

The technologies based on aerobic granular biomass are relatively recent and their advantages compared to the Conventional Activated Sludge (CAS) process make them a feasible alternative to implement. One of these benefits is the lower sludge production compared to the CAS process (Campos *et al.*, 2009). However the aggregation of the biomass in granules and the high content of insoluble Extracellular Polymeric Substances (EPS) in the granule shell (Wang *et al.*, 2005) could limit its treatment by anaerobic digestion, since it is known that the EPS are relatively recalcitrant to anaerobic and aerobic digestion by nature (Carrère *et al.*, 2010).

The results obtained in Chapter 6 for the anaerobic digestion of Aerobic Granular Sludge (AGS) in discontinuous experiments indicated that its biodegradability with and without thermal pre-treatment is similar to that corresponding to WAS. At the moment no information regarding a research work about the continuous anaerobic digestion of AGS is available.

## 7.2. OBJECTIVE

The objective of this work is to study the feasibility of the application of the anaerobic digestion for the treatment of AGS. Different compositions of the feeding media were evaluated in terms of methane production, BioDegradability (BD) and solids reduction: raw AGS, thermal pre-treated AGS and the mixture of thermal pre-treated AGS with raw PS.

Microbial analysis applying the Fluoresecence *In Situ* Hybridization (FISH) technique were performed to evaluate the possible influence of the type of feeding on the development of different microbial populations inside the anaerobic reactor.

## 7.3. MATERIALS AND METHODS

### 7.3.1. Aerobic Granular Sludge (AGS) samples

The AGS was collected from a pilot plant SBR (100 L), treating the liquid fraction of pig slurry and corresponding to sludge AGS1 from Chapter 6. The AGS was collected along several operational days to accumulate the sufficient amount of solids to perform the experiments in the anaerobic digester. In order to remove the highest quantity of water and to concentrate the sludge, the sample was left to settle and the supernatant was removed. The PS used to co-digest with the AGS was collected from the urban WWTP of Vigo (Pontevedra, Spain). To avoid a possible degradation of the feeding both AGS and PS were stored at 4 ºC.

### 7.3.2. Anaerobic sludge inoculum

The inoculum utilized to start-up the reactor was flocculent anaerobic sludge from the anaerobic sludge digester of the urban WWTP of Vigo, which treated a mixture of PS and WAS at mesophilic range. The Specific Methanogenic Activity (SMA) of the flocculent anaerobic sludge was of 1.1 g COD-$CH_4$/g $VS_{inoculum}$·d.

### 7.3.3. Thermal pre-treatment

The thermal pre-treatment of the AGS was performed in an autoclave (Raypa Stericlav-75) at 133 ºC during 20 minutes, although the total time necessary to reach the desired temperature together with that needed to discharge the sample prolonged the treatment in 60 more minutes. The temperature of pre-treatment was chosen considering the enhancement in the anaerobic BD of the AGS sample obtained in the batch experiments of Chapter 6, where the adequate temperature seems to be around 140 ºC, and also taking into account the thermal pre-treatment temperature used for WAS in a continuous anaerobic digester in previous works (135 ºC, Bougrier *et al.*, 2007).

### 7.3.4. Experimental set-up

A Continuously Stirred Tank Reactor (CSTR) with a useful volume of 5 L and provided with a thermostatic jacket was used (Figure 7.1). The anaerobic reactor was operated in fed-batch mode and a volume of 500 mL was removed and fed each 2 days. The feeding and the effluent withdrawal were manually done to avoid the use of peristaltic pumps which could disintegrate the granules. The temperature was keep at 35 ºC by means of a thermostatic bath. The complete mixture inside the reactor was achieved by means of a mechanical stirrer with a rotating speed of 100 rpm and the solids retention time (SRT) was fixed at 20 days. The volume of produced biogas was measured by means of a water displacement device (Veiga *et al.*, 1990).

(A) (B)

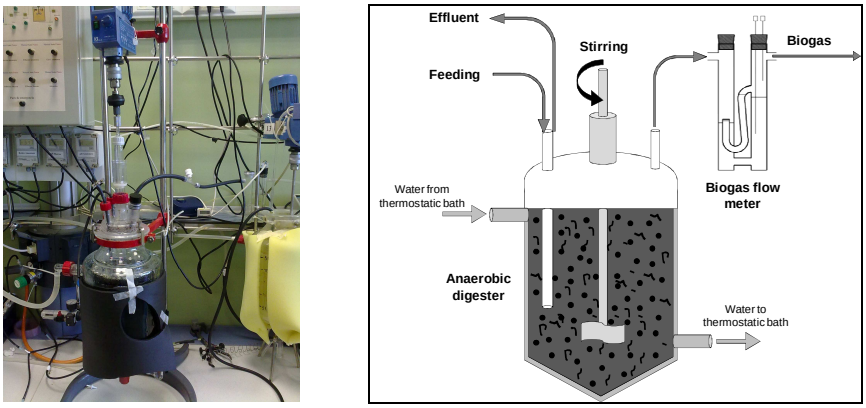


**Figure 7.1.** (A) Image of the CSTR and (B) layout of the experimental set-up.

### 7.3.5. Strategy of operation

The anaerobic digester was operated in three different stages corresponding to the different substrates used to feed the reactor (Table 7.1). Firstly, in Stage I (0-309 days), the raw AGS was digested in order to evaluate the anaerobic BD of this type of sludge. This stage comprised three different substages: Stage IA corresponded to the start up of the reactor (0-35 days) when the applied Solids Loading Rate (SLR) was of 0.4 g $VS_{fed}$/L·d, Stage IB (36-205 days) corresponded to the operation at a stable SLR around 1.0 g $VS_{fed}$/L·d and Stage IC (206-309 days) is a period of unstable operational conditions with a variable SLR (between 0.7 and 1.3 g $VS_{fed}$/L·d). During Stage II (310-416 days) the AGS was submitted to a thermal pre-treatment to enhance its BD and the production of biogas. Finally in Stage III (417-486) the pre-treated AGS was mixed with the PS in a proportion of 50%-50% (VS/VS) to simulate the operational conditions in a WWTP.

**Table 7.1.** Operational conditions and characteristic parameters of the feeding of the anaerobic digester.

| Parameter | Stage I | | | Stage II | Stage III |
|---|---|---|---|---|---|
| | IA | IB | IC | | |
| Days of operation | 0-35 | 36-205 | 206-309 | 310-416 | 417-486 |
| *Operational conditions* | | | | | |
| SLR (g $VS_{fed}$/L·d) | 0.4 ± 0.1 | 1.0 ± 0.1 | 0.7–1.3 | 0.6 – 1.3 | 0.6 ± 0.1 |
| OLR (g $COD_T$/L·d) | 0.4 ± 0.1 | 1.2 ± 0.1 | 1.4–2.4 | 0.8 – 2.1 | 1.2 ± 0.1 |
| *Feeding characteristics* | | | | | |
| $COD_T$ (g/L) | 7.7 ± 1.9 | 24.8 ± 2.3 | 27.5–47.2 | 28.6 ± 7.7 | 24.9 ± 2.0 |
| $COD_S/COD_T$ (%) | 1.9 ± 0.8 | 7.4 ± 3.9 | 13.1 ± 2.1 | 35.0 ± 6.2 | 27.8 ± 4.9 |
| TS (g/L) | 9.2 ± 2.3 | 21.1 ± 1.6 | 15.7–27.0 | 22.4 ± 5.6 | 16.6 ± 2.3 |
| VS/TS (%) | 92.3 ± 1.6 | 90.9 ± 0.6 | 92.7 ± 0.4 | 87.3 ± 2.9 | 74.9 ± 5.2 |
| $COD_T$/VS (g/g) | 1.0 ± 0.4 | 1.2 ± 0.1 | 1.9 ± 0.1 | 1.5 ± 0.3 | 2.0 ± 0.6 |
| VFA (g/L) | ND | ND | 1.0 ± 0.3 | 0.7 ± 0.6 | 1.5 ± 0.4 |
| Average diameter (mm) | 1.3 ± 0.1 | 1.6 ± 0.1 | 1.4 ± 0.2 | – | – |

SLR: Solids Loading Rate
OLR: Organic Loading Rate
VFA: Volatile Fatty Acids
ND: Not detected

### 7.3.6. Calculations

The free ammonia ($NH_3$) concentration in the reactor was estimated according to Anthonisen *et al.* (1976). The proteins were calculated from organic nitrogen composition and the carbohydrates were estimated as the remaining fraction of VS after subtracting the proteins and lipids content (Álvarez *et al.*, 2010). The BD was determined according to Mottet *et al.*

(2010) by dividing the production of methane in N-$mL_{CH4}$/g $COD_{fed}$ by the maximum theoretical $CH_4$ production in standard conditions (350 N-$mL_{CH4}$/g COD at 1 atm and 0 ºC). The Potential Methanogenic Activity (PMA) in terms of g COD-$CH_4$/g $VS_{reactor}$·d was determined as the slope of the curve of methane production obtained after fed the reactor. For further information about the calculations see Chapter 2.

### 7.3.7. Analytical methods

Total Solids (TS), Volatile Solids (VS), Total Alkalinity (TA) and ammonium ($NH_4^+$) concentrations and pH value were determined according to the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The soluble Chemical Oxygen Demand ($COD_S$) was determined by a semi-micro method (Soto *et al.*, 1989) and the total COD ($COD_T$) was determined according to the closed reflux colorimetric method (APHA-AWWA-WPCF, 2005). The biomass composition (C, H, N, O and S) was obtained by an elemental analysis technique based on the complete and instantaneous oxidation (combustion) of the sample and determination of the gases composition from the combustion through a thermal conductivity detector (model CHNS FISONS EA 1108 for C, H, N and S; model CARLO ERBA EA 1108 for oxygen). Lipids concentration was measured by solvent extraction using petroleum ether. The size of the granules was measured by using an image analysis procedure with a stereomicroscope (Stemi 2000-C, Zeiss). The Volatile Fatty Acids (VFAs) concentration was determined by liquid chromatography (HP, 5890) and the percentage of methane in the biogas was measured collecting 1 mL of sample with a gas-tight syringe and injecting it into the gas chromatograph (HP, 5890 Series II). The SMA of the anaerobic sludge inoculum was determined according to the methodology proposed by Soto *et al.* (1993).

Active microbial populations were identified by the FISH technique. Fresh biomass samples were disrupted and fixed with 4% paraformaldehyde according to the procedure described by Amann *et al.* (1995). Hybridization was performed at 46 ºC for 90 minutes, adjusting formamide concentrations for each probe at the percentages shown in Table 7.2. The used probes for *in situ* hybridization were 5' labelled with the fluorochromes FITC, Cy3 or Cy5. The samples were observed by an acquisition system (Coolsnap, Roper Sicientific Photometrics) coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany). Quantification of microbial populations was based on the procedure published by Crocetti *et al.* (2002). For each hybridization experiment the quantification was performed by comparison of the positive area, obtained with a certain FISH probe, with the area corresponding to the control sample, after application of the DAPI dye which bonds the DNA and using the program of image analysis Image ProPlus®.

**Table 7.2.** Probes used for FISH analysis of the biomass samples and corresponding 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] |
| MA450 | ATCCAGGTACCGTCATTATC | 20 | Type II methanotrophs (*Methylosinus/Methylocystis* spp.) | [4] |
| BET42a | GCCTTCCCACTTCGTTT | 35 | *Betaproteobacteria* | [3] |
| Competitor | GCCTTCCCACATCGTTT | | | |
| AQS997 | CTCTGGTAACTTCCGTAC | 35 | *Curvibacter* | [5] |
| Competitor | CTCTGGCAACTTCCGTAC | | | |
| Competitor | CTCTGGTCACTTCCGTAC | | | |
| CTE | TTCCATCCCCCTCTGCCG | 20 | *Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp., *Aquaspirillum* spp. | [6] |
| MZ1 | TCTGCCGTACTCTAGCCTT | 45 | *Thauera* spp. *mzt1t* | [7] |
| GAM42a | GCCTTCCCACATCGTTT | 35 | *Gammaproteobacteria* | [3] |
| Competitor | GCCTTCCCACTTCGTTT | | | |
| MG705 | CTGGTGTTCCTTCAGATC | 20 | Type I methanotrophs | [4] |
| MG84 | CCACTCGTCAGCGCCCGA | 20 | Type I methanotrophs | [4] |
| DELTA495a | AGTTAGCCGGTGCTTCTT | 35 | Most *Deltaproteobacteria* | [8] |
| Competitor | AGTTAGCCGGTGCTTCTT | | | |
| SRB385 | CGGCGTCGCTGCGTCAGG | 35 | Most *Desulfovibrionales* and other Bacteria | [1] |
| DSBAC357 | CCATTGCGCAAAATTCCTCAC | 35 | Most *Desulfobacteraceae* and *Syntrophobacteraceae* | [9] |
| Competitor | CCATTGCGCAAAATTCCCCAC | | | |
| Competitor | CCATTGCGCAAAATCCCTCAC | | | |
| CFB562 | TACGYWCCCTTTAAACCCA | 30 | Subgroup of *Bacteroidetes* | [10] |
| CF319ab | TGGTCCGTGTCTCAGTAC | 35 | Most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria* | [11] |
| CFX1223 | CCATTGTAGCGTGTGTGTMG | 35 | Phylum *Chloroflexi* | [12] |
| GNSB941 | AAACCACACGCTCCGCT | 35 | Phylum *Chloroflexi* | [13] |
| HOL1400 | TTCGTGATGTGACGGGC | 20 | *Acidobacteria* | [14] |
| SYNM700 | ACTGGTNTTCCTCCTGATTTCTA | 30 | Mesophilic members of the family *Syntrophomonadaceae* | [15] |
| ARC915 | GTGCTCCCCCGCCAATTCCT | 20 | *Archaea* | [16] |
| MB1174 | TACCGTCGTCCACTCCTTCCTC | 45 | *Methanobacteriales* (minus *Methanothermus*) | [17] |
| MS821 | CGCCATGCCTGACACCTAGCGAGC | 40 | *Methanosarcina* | [17] |
| MX825 | TCGCACCGTGGCCGACACCTAGC | 50 | Some *Methanosaetaceae* | [17] |
| EUK516 | ACCAGACTTGCCCTCC | 25 | *Eukarya* | [1] |

[a] [1] Amann *et al.* (1990); [2] Daims *et al.* (1999); [3] Manz *et al.* (1992); [4] Eller *et al.* (2001); [5] Thomsen *et al.* (2004); [6] Schleifer *et al.* (1992); [7] Lajoie *et al.* (2000); [8] Loy *et al.* (2002); [9] Luecker *et al.* (2007); [10] O'Sullivan *et al.* (2002); [11] Manz *et al.* (1996); [12] Bjornsson *et al.* (2002); [13] Gich *et al.* (2001); [14] Meisinger *et al.* (2007); [15] Hansen *et al.* (1999); [16] Stahl and Amann (1991); [17] Raskin *et al.* (1994).

A more detailed description of all the used analytical methods is provided in Chapter 2.

## 7.4. RESULTS AND DISCUSSION

### 7.4.1. Anaerobic digester performance stability

The concentrations of VFA, TA, $NH_3$ and the pH value were determined regularly as indicators of the digester operation stability (Figure 7.2). The VFA concentration did not exceed the value of 0.16 g/L (as acetic acid) and it remained below the recommended range for anaerobic methanogens (1.0-1.5 g/L; Erden and Filibeli, 2010). The TA was always higher than 2.5 g $CaCO_3$/L and the pH ranged between 7.2 and 7.6, which indicated that these parameters were also within the favourable ranges. The free ammonia concentration was between 0.02 and 0.05 g $NH_3$-N/L which was lower than the reported inhibitory threshold concentrations of 0.06 g $NH_3$-N/ L for methanogenic bacteria (Bhattacharya and Parkin, 1989).

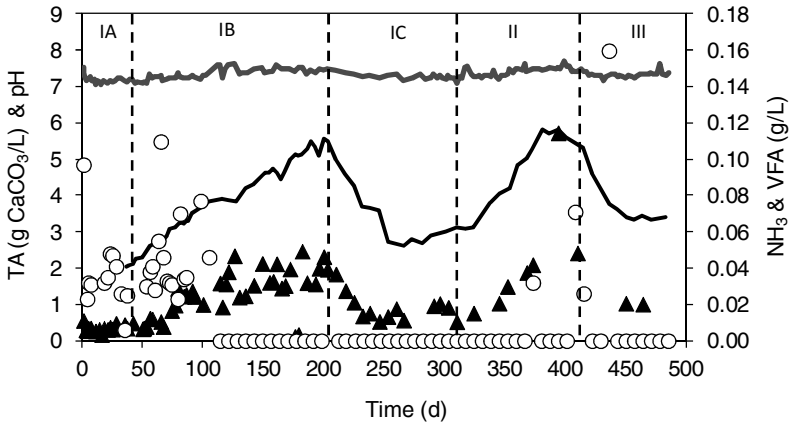


**Figure 7.2.** Profile of the TA (-), pH (-), $NH_3$ (▲) and VFA (○ ) in the anaerobic reactor.

### 7.4.2. Anaerobic digestion and methane production

The SLR, applied to the anaerobic digester, and the methane production, related to the $VS_{fed}$, are presented in Figure 7.3. The digester was fed during Stage I with raw AGS and started-up with a SLR of 0.4 g $VS_{fed}$/L·d to acclimatise the inoculum. The methane production during the first days of operation was high around 300 N-$mL_{CH4}$/g $VS_{fed}$, probably due to the presence of rests of biodegradable organic matter in the inoculum. Then the SLR was progressively increased up to 1.0 g $VS_{fed}$/L·d (Stage IB). The average methane production along this stage was of 208 N-$mL_{CH4}$/g $VS_{fed}$, reaching at the end of this period a value of 250 N-$mL_{CH4}$/g $VS_{fed}$. Along Stage IC the SLR applied varied between 0.7 and 1.3 g $VS_{fed}$/L·d and the average methane production was only 150 N-$mL_{CH4}$/g $VS_{fed}$. This decrease could be due to the fact that the feeding was characterized by the increase of straw content coming from the pig slurry. This straw was not quantified but it could be appreciated incrusted to the surface of the granules fed (Figure 7.4). Furthermore the higher $COD_T$/VS ratio of the feeding along Stage IC (Table 7.1) in comparison with the previous stage indicated that part of the organic content on this feeding was not easily biodegradable. Inside the anaerobic digester this straw formed a

matted scum layer difficult to break up in the top of the reactor and, probably, it severely affected the biogas production due to the flotation and poor mixing of fibrous materials contained in the straw (Cui *et al.*, 2011). For this reason the Stage IC was considered not representative of a period of stable operation conditions. The different characteristics of the feeding in Stage IC also provoked a decrease in the TA inside the reactor, although the pH and VFAs concentration were not affected and even the free ammonia concentration decreased (Figure 7.2).

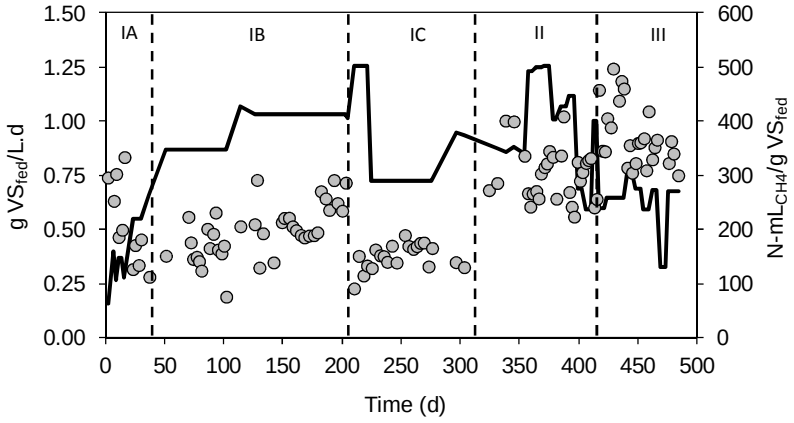


**Figure 7.3.** Profile of the SLR as g $VS_{fed}$/L·d (**–**) fed to the anaerobic digester and the methane production measured as N-$mL_{CH4}$/g $VS_{fed}$ (●) along the operation time.

**Figure 7.4.** Picture of the AGS fed to the anaerobic digester in Stage IC. The size bar corresponds to 2 mm.

In Stage II the reactor was fed with AGS thermally pre-treated at 133 ºC and the applied SLR ranged between 0.6 and 1.3 g $VS_{fed}$ /L·d. The methane production during this stage was around 309 N-$mL_{CH4}$/g $VS_{fed}$, which implied an enhancement respect to the raw AGS (Stage IB) of 49%. This enhancement was due to the solubilisation of the organic matter compounds provoked by the thermal pre-treatment (the fraction of $COD_S$ increased, Table 7.1), as occurred in Chapter 6 for the batch experiments.

In order to compare the results obtained in batch tests (Chapter 6) and in the anaerobic digester (this study) for AGS sample with and without thermal pre-treatment a summary of the results is presented in Table 7.3. The methane production and anaerobic BD treating raw AGS was similar for both conditions, taking into account that the standard deviation of the results in the fed-batch anaerobic digester was higher. The differences on the results could be due to the heterogeneity of the AGS samples. Furthermore it is important to point out that the results obtained after the thermal pre-treatment were very similar in both systems (batch tests and fed-batch anaerobic digester).

**Table 7.3.** Comparison of batch tests and fed-batch anaerobic digester for AGS without (raw) and with thermal pre-treatment (pre-treated).

| Type of feeding | N-mL$_{CH4}$/g VS$_{fed}$ | N-mL$_{CH4}$/g COD$_{fed}$ | BD (%) |
|---|---|---|---|
| *Raw AGS* | | | |
| Batch tests [a] | 169 ± 7 | 116 ± 5 | 33 ± 1 |
| Fed-batch digester [b] | 208 ± 51 | 154 ± 37 | 44 ± 11 |
| *Pre-treated AGS* | | | |
| Batch tests [a] | 308 ± 14 | 209 ± 10 | 60 ± 3 |
| Fed-batch digester [b] | 309 ± 58 | 203 ± 31 | 58 ± 9 |
| *(Pre-treated AGS) + PS* | | | |
| Fed-batch digester [b] | 343 ± 40 | 186 ± 17 | 53 ± 5 |

[a] Data from Chapter 6. Pre-treated sludge: 140 ºC, 20 minutes.
[b] Data from this work. Pre-treated sludge: 133 ºC, 20 minutes.

In Stage III the feeding of the anaerobic digester was composed by a mixture of pre-treated AGS and raw PS. Although the applied OLR was similar to that fed in Stage IB (1.2 g COD$_{fed}$/L·d, Table 7.1) the applied SLR was of 0.6 g VS$_{fed}$ /L·d due to the lower concentration of VS in the PS. The methane production along this stage referred to the solids fed was around 343 N-mL$_{CH4}$/g VS$_{fed}$ and higher than the value obtained with only pre-treated AGS (309 N-mL$_{CH4}$/g VS$_{fed}$, Stage II), which coincided with previous works digesting WAS alone and WAS mixed with PS (Bouallagui *et al.*, 2010). However if the methane production is referred to the COD fed, this was worse in Stage III than in Stage II (Table 7.3). This difference was due to the higher COD$_T$/VS ratio of the mixture pre-treated AGS and raw PS compared to that of the pre-treated AGS (Table 7.1).

### 7.4.3. Potential Methanogenic Activity (PMA)

The reactor was fed every two days with an amount of sludge equal to that previously removed as effluent. The fed-batch mode operation of the reactor was evaluated by means of the Potential Methanogenic Activity (PMA) calculated from the biogas production along the time after each feeding and divided by the amount of biomass in the reactor (Figure 7.5). The slope

of the curves describing the cumulative methane production, which followed a linear trend indicating a zero order kinetic, was obtained. In Stage I with raw AGS the PMA was of 0.23 g COD-$CH_4$/g $VS_{reactor}$·d, that was a very low value compared to the SMA of the inoculum (1.1 g COD-$CH_4$/g $VS_{inoculum}$·d). In Stages II (with the thermal pre-treated AGS) and III (mixture of pre-treated AGS and raw PS) two different rates of biogas production were observed. During the first 21 hours after the feeding addition the PMA was of 0.87 and 0.94 g COD-$CH_4$/g VS·d in Stages II and III, respectively. These values are close to the initial SMA obtained with the inoculum, which would correspond mainly to the anaerobic digestion of the soluble COD. Along the 27 hours left before the next feeding supply the PMA was of 0.20 and 0.25 g COD-$CH_4$/g $VS_{reactor}$·d in Stages II and III, respectively. These values were similar to those observed in Stage I with raw AGS, which presumably corresponded to the degradation of the particulate COD.

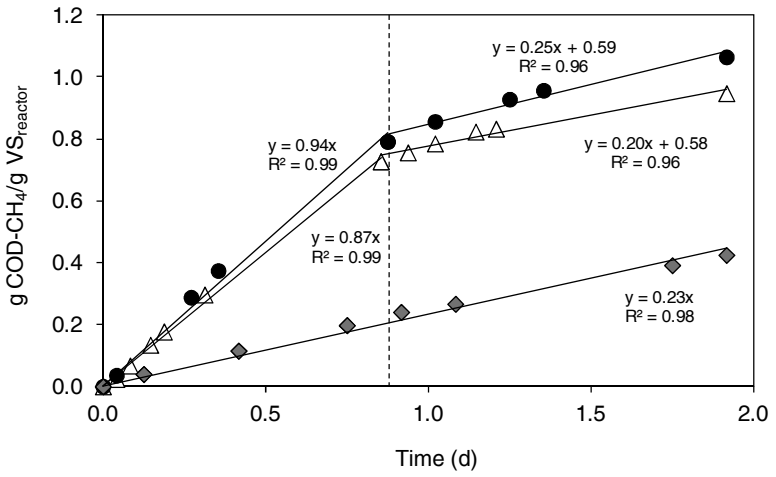


**Figure 7.5.** Cumulative methane production for a batch of feeding in Stage IB (◆), Stage II (△) and Stage III (●).

### 7.4.4. Solids removal

In Figure 7.6 the VS, proteins and carbohydrates removal efficiencies are represented for each operational stage (the lipids content in the sludge samples was very low and under the detected level of the analytical method). The results have been obtained after the stabilisation of the reactor in each stage (60 days after change of the feeding).

The solids removal in terms of VS was around 32% for the raw AGS (Stage IB). The thermal pre-treatment improved the solids removal to 47% (Stage II), which supposed an enhancement of 47%. The solids removal of the mixture pre-treated AGS with raw PS (Stage III) was 55%, although the VS concentration fed was lower than in Stage II due to the high $COD_T$/VS ratio of the mixture caused by the higher content of mineral solids in the PS. Bouallagui *et al.* (2010) observed that the anaerobic digestion of a mixture of PS and WAS had a better solids removal efficiency when the proportion of biological sludge was lower than that of the PS (20:80). In this work the mixture of both sludge types in a proportion of 50:50 (Stage III)

was chosen in order to use a similar value than that normally used in the WWTPs. However this proportion could be different in the hypothetical case of a WWTP operated with an aerobic granular system where the amount of produced biological sludge is expected to be around 35% lower than in a CAS process (Campos *et al.*, 2009). In this situation the AGS:PS ratio would be around 40:60, which could be beneficial for the solids removal in the anaerobic digester as shown from Figure 7.6.

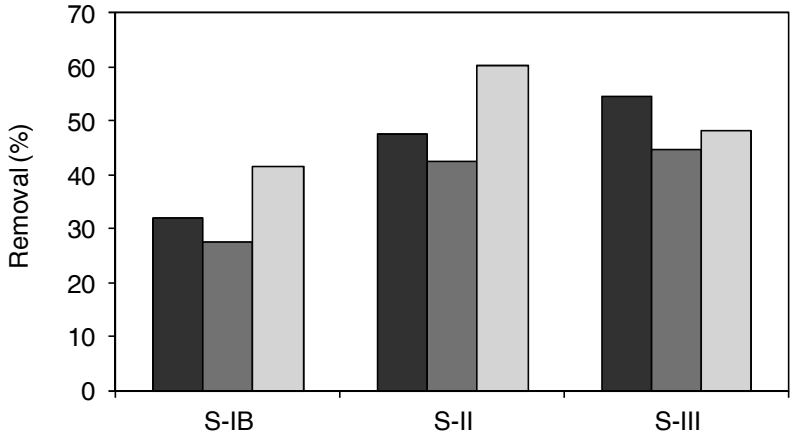


**Figure 7.6.** Removal percentages of VS (■), proteins (■) and carbohydrates (■) for the different operational stages.

To determine which compounds were preferentially degraded during AGS anaerobic digestion, the concentration of proteins and carbohydrates in the feeding and in the outlet of the reactor were also studied. The percentages of proteins and carbohydrates removal were for raw AGS around 28% and 42%, respectively (Stage IB) and for the pre-treated AGS of 43% and 60%, respectively (Stage II). This increase in the organic compounds removal can be correlated to the disintegration of the aerobic granules due to the thermal pre-treatment that possibly led to an EPS destructuration promoting the solubilisation of extracellular proteins and carbohydrates and increasing the soluble COD (Bougrier *et al.*, 2008).

The removal efficiencies of proteins and carbohydrates with the mixture of pre-treated AGS and raw PS (Stage III) were of 45% and 48%, respectively, that in comparison to the Stage II (only pre-treated AGS) supposed a decrease in the carbohydrates removal, maybe related to the composition of the PS.

### 7.4.5. Anaerobic digestion of AGS vs. WAS

The methane production and removal efficiencies obtained in the present work with AGS are compared in Table 7.4 to those achieved during the anaerobic digestion of WAS carried out under similar operational conditions: SRT of 20 days, digestion temperature of 35 °C, OLR around 1 g COD/L·d and with or without thermal pre-treatment (Bougrier *et al.*, 2007).

**Table 7.4.** Comparison between AGS and WAS anaerobic digestion without (raw) and with thermal pre-treatment (pre-treated).

| Type of feeding | N-mL$_{CH4}$/g VS$_{fed}$ | BD (%) | VS$_{removed}$ (%) | Proteins$_{removed}$ (%) | Carbohydrates$_{removed}$ (%) |
|---|---|---|---|---|---|
| *Raw sludge* | | | | | |
| AGS [a] | 208 ± 51 | 44 ± 11 | 32 | 28 | 42 |
| WAS [b] | 254 ± 31 | 50 ± 3 | 39 | 35 | 56 |
| *Pre-treated sludge* | | | | | |
| AGS [a] | 309 ± 58 | 58 ± 9 | 47 | 43 | 48 |
| WAS [b] | 285 ± 22 | 54 ± 2 | 41 | 43 | 68 |

[a] This work. Pre-treated sludge: 133 ºC, 20 minutes.
[b] Bougrier *et al.* (2007). Pre-treated sludge: 135 ºC, 30 minutes.

The results of methane production (N-mL$_{CH4}$/g VS$_{fed}$) and BD obtained for AGS are similar than those of reported by Bougrier *et al.* (2007) for WAS, although the values of the standard deviations were higher in this work. The anaerobic BD of AGS (44%) is inside the typical range of 30-50% considered for a WAS (Mottet *et al.*, 2010). So the BD of AGS obtained in the continuous fed-batch anaerobic digester confirms the results obtained in the batch experiments in Chapter 6, that the anaerobic digestion of AGS is comparable to that of WAS. This is an interesting result taking into account that the biomass in form of granules could be expected to be more difficult to digest due to its small surface to volume ratio and its higher EPS content, which could negatively affect the rate of the hydrolysis step. Therefore the anaerobic digestion could be considered as a feasible option to reduce solids coming from an aerobic granular reactor.

In this study the AGS with an average diameter around 1.6 mm presented a comparable anaerobic BD to that of the WAS with a floc particle size around 35-50 µm (Bougrier *et al.*, 2006). The fact that the anaerobic digestion only supposed a partial disintegration of the granule while its main structure remained almost intact could be observed comparing the visual aspect of the granules before (Figure 7.7A) and after (Figure 7.7B) the anaerobic digestion.

The thermal pre-treatment of the sludge samples exerted a more beneficial effect on the enhancement of the BD, the VS and proteins removals in the case of the AGS (32%, 47% and 54%, respectively) than in the case of the WAS (8%, 5% and 23%, respectively), which could be due to the higher COD solubilisation of the former (Bougrier *et al.*, 2008). In this study the percentage of COD solubilisation with the thermal pre-treatment at 133 ºC respect to the untreated AGS was of 28% (calculated from the data of Table 7.1). This value was similar to the obtained values in Chapter 6, while on the reported work of Bougrier *et al.* (2007) using WAS this percentage of improvement was only of 2% at 135 ºC. The enhancement of carbohydrates removal was slightly lower in the case of AGS (14%) than in the case of WAS (21%).

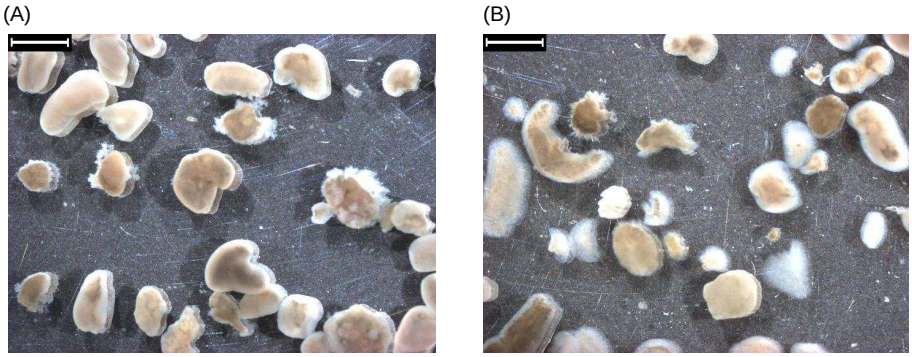


**Figure 7.7.** Images of aerobic granules before (A) and after (B) the anaerobic digestion (Stage IB). The size bar corresponds to 2 mm.

### 7.4.6. Microbial populations

The FISH technique was applied to characterize the main populations present in the biomass samples collected from the anaerobic reactor in each operational stage and to evaluate if the different type of feeding produced a shift in the microbial populations (Table 7.5). The main features observed from the samples indicated that the *Archaea* domain is more abundant in comparison with the *Bacteria* domain and that the abundance of both domains decreased in Stage III in comparison with the previous stages (Figure 7.8), probably belonged with the presence of a higher quantity of inert solids in the feeding due to the addition of PS. Members of the *Fungi* domain were not detected.

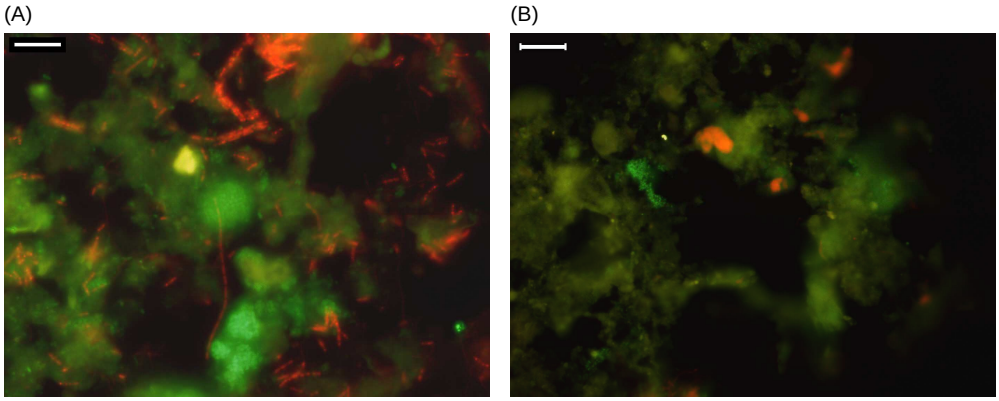


**Figure 7.8.** FISH images of anaerobic sludge with probes EUB338mix (FITC) and ARC915 (Cy3) in: (A) Stage IB and (B) Stage III. The bar indicates 10 and 25 μm for images (A) and (B), respectively.

**Table 7.5.** Relative abundance of microorganisms detected with the FISH probes in the different operational stages.

| Probe | Target organisms | Stage IB (Day 71) | Stage II (Day 399) | Stage III (Day 484) |
|---|---|---|---|---|
| $EUB338_{mix}$ | *Bacteria* domain | ++ | ++ | + |
| ALF1b | *Alphaproteobacteria* | + | + | + |
| MA450 | Type II methanotrophs (*Methylosinus/Methylocystis* spp.) | - | - | - |
| BET42a | *Betaproteobacteria* | ++ | ++ | + |
| AQS997 | *Curvibacter* | - | - | - |
| CTE | *Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp., *Aquaspirillum* spp. | + | + | + |
| MZ1 | *Thauera* spp. *mzt1t* | + | - | + |
| GAM42a | *Gammaproteobacteria* | + | + | + |
| MG705+MG84 | Type I methanotrophs | - | - | - |
| DELTA495a | Most *Deltaproteobacteria* | + | + | + |
| SRB385 | Most *Desulfovibrionales* and other Bacteria | - | - | - |
| DSBAC357 | Most *Desulfobacteraceae* and *Syntrophobacteraceae* | - | - | - |
| CFB562+CF319ab | Subgroup of *Bacteroidetes*, most *Flavobacteria*, some *Sphingobacteria* | ++ | + | + |
| CFX1223+GNSB941 | Phylum *Chloroflexi* | + | + | - |
| HOL1400 | *Acidobacteria* | - | - | - |
| SYNM700 | Mesophilic members of the family *Syntrophomonadaceae* | + | + | + |
| ARC915 | *Archaea* | +++ | +++ | ++ |
| MB1174 | *Methanobacteriales* (minus *Methanothermus*) | - | - | - |
| MS821 | *Methanosarcina* | - | - | - |
| MX825 | Some *Methanosaetaceae* | + | + | + |
| EUK516 | *Eukarya* | - | - | - |
| MY1574 | *Eumycota* (fungi) | - | - | - |

Note: - not detected, + present, ++ abundant, +++ very abundant.

***Bacteria domain***

For the identification of members of the *Bacteria* domain the application of the probe $EUB338_{mix}$ and the DAPI dye indicated that there was a high quantity of bacteria and they grouped in clusters. The probe $EUB338_{mix}$ in combination with probe BET42a showed that an important part of the observed bacteria (80-90%) belonged to *Betaproteobacteria* class. The signal obtained with ALF1b, GAM42a and DELTA495a probes compared with that of DAPI indicated the presence of a low percentage of *Alfa*- (<1%), *Gamma*- (≈3%) and *Delta*- (<1%) *Proteobacteria*. The higher quantity of *Betaproteobacteria* in comparison with *Gammaproteobacteria* can be observed in Figure 7.9 for the Stage IB.

The probe MA450, specific for the *Methylocystaceae* (Type II methanotrophs) from the genus *Alfaproteobacteria*, was tested and did not provide positive results in any stage of operation.

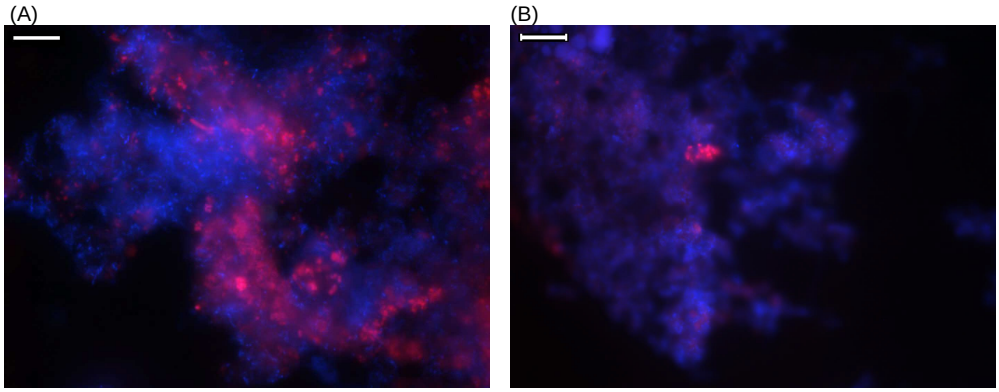


**Figure 7.9.** FISH images of anaerobic sludge in Stage IB. (A) BET42a (Cy3) and DAPI. (B) GAM42a (Cy3) and DAPI. The bar indicates 10 and 25 μm for images (A) and (B), respectively.

Probes AQS997, CTE and MZ1 which target the group of *Betaproteobacteria* were tested. The probe AQS997 that identify microcolonies of a dominant denitrifying bacterium in activated sludge (Genus *Curvibacter*) did not provide positive results in any of the three stages of operation. The application of probe CTE, to identify the family *Comamonadaceae*, produced a positive signal in all samples (Figure 7.10) which indicated that it was the main *Betaproteobacteria* present in the anaerobic digester. Ariesyady *et al.* (2007) in a full-scale anaerobic sludge digester, which treated the excess sludge of an urban WWTP in the mesophilic range (40 ºC), observed that the most part (90%) of the *Betaproteobacteria* were affiliated with *Comamonadaceae*. The probe MZ1 to identify bacteria from the genus *Thauera* only showed a positive result in stages I and III, but with a low quantity in comparison with the total population of *Betaproteobacteria*. Rivière *et al.* (2009) also found this type of bacteria in sludge samples from seven different full-scale anaerobic sludge digesters (treating municipal sewage sludge) and more precisely the species *T. phenylacetica* and *T. Aminaromatica*.

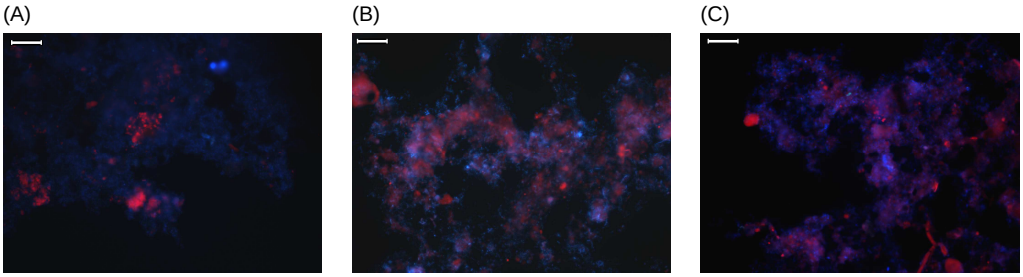


**Figure 7.10.** FISH images of anaerobic sludge with probe CTE (Cy3) and DAPI in: (A) Stage IB, (B) Stage II and (C) Stage III. The bar indicates 25 μm.

The family of *Gammaproteobacteria* was tested with the combined probes MG705 and MG84, that are specific for the genus *Methylococcaceae* (Type I methanotrophs) and the family of *Deltaproteobacteria* with the probes SRB385 and BSBAC357, that are specific for most

*Desulfovibrionales* and most *Desulfobacteraceae* and *Syntrophobacteraceae*, respectively. In all the cases the FISH analysis did not provide positive hybridization.

The combined probes CFB562 and CF319ab were applied to identify the bacteria corresponding to the *Bacteroidetes* group. The results showed a higher presence of this group on Stage I (Figure 7.11A) and in a minor proportion in Stages II and III. *Bacteroidetes* are known to be proteolytic bacteria (Kindaichi *et al.*, 2004), which firstly carry out the degradation of proteins and are able to ferment amino acids to acetate (Rivière *et al.*, 2009). Ariesyady *et al.* (2007) observed that the *Bacteroidetes* was one of the major bacterial phyla (21%) in anaerobic reactor treating the excess sludge of urban WWTP.

The *Chloroflexi* group was detected by the application of a combination of CFX1223 and GNSB941 probes in Stages I and II (Figure 7.11B), although in a minor proportion in this last stage. No positive hybridization was detected for Stage III. Rivière *et al.* (2009) and Ariesyady *et al.* (2007) observed that this group represented around 32 and 14%, respectively, of the total bacteria domain in anaerobic sludge samples coming from the digestion of municipal sewage sludge, and several studies showed their potential role in the degradation of carbohydrates (Kindaichi *et al.*, 2004; Ariesyady *et al.*, 2007). The lower quantity of the phyla *Chloroflexi* in Stage II in comparison with Stage I and its absence in Stage III indicated that a wash out or inactivation of this bacteria occurred along the reactor operational period. This fact could be related to the application of the thermal pre-treatment of the AGS fed to the anaerobic digester. Héry *et al.* (2010) studied the microbial population of activated sludge coming from the treatment of domestic wastewater and observed that, after submit it to a thermal treatment at 65 ºC along 85 hours, the *Chloroflexi* bacteria disappeared. This suggests that the presence of *Chloroflexi* bacteria in the anaerobic digester came from the AGS used as feeding and that the application of the thermal pre-treatment provoked their disappearance.

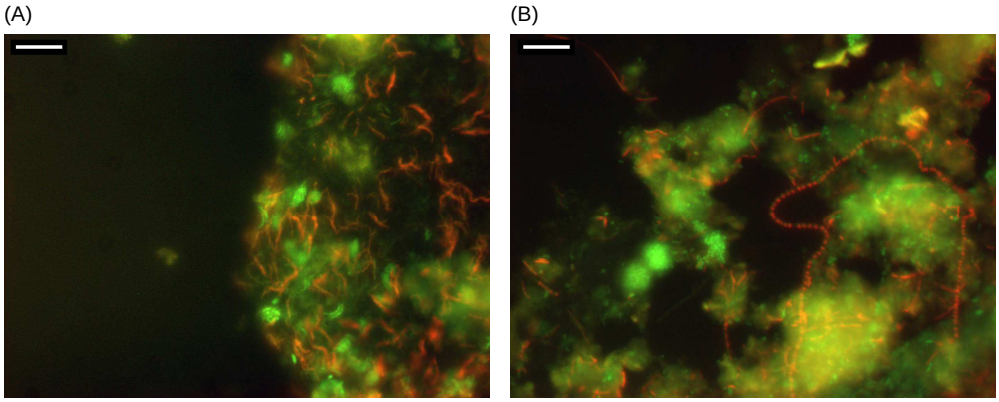


**Figure 7.11.** FISH images of the anaerobic sludge in Stage IB. (A) CFB562+CF319ab (Cy3) and $EUB338_{mix}$ (FITC). (B) CFX1223+GNSB941 (Cy3) and $EUB338_{mix}$ (FITC). The bar indicates 10 μm.

The *Acidobacteria* presence was tested with the probe HOL1400, but no positive results were obtained. This is in accordance with other studies (Ariesyady *et al.*, 2007; Rivière *et al.*, 2009; Krakat *et al.*, 2011) that showed that it is not a common group on anaerobic sludge.

The probe SYNM700 was used to identify the mesophilic members of the family *Syntrophomonadaceae* belonging to the phyla *Firmicutes*, that are fatty-acid oxidizing bacteria (Shigematsu *et al.*, 2006), obtaining a positive result in all stages although in small amounts. Previous works show that this is one of the main bacteria domain detected in anaerobic sludge (Ariesyady *et al.*, 2007; Rivière *et al.*, 2009; Krakat *et al.*, 2011) and that it is present in thermal treated activated sludge but not in untreated ones (Héry *et al.*, 2010).

***Archaea domain***

The probe ARC915 that identifies the populations from the *Archaea* domain indicated the presence of large amounts of these organisms compared with the *Bacteria* domain. Inside this domain the probes MB1174, MS821 and MX825 that identify *Methanobacteriaceae*, *Methanosarcinaceae* and *Methanosaetaceae* bacteria, respectively, were tested. The results of the FISH hybridization indicated that only *Methanosaetaceae* was present in the three operational stages (Figure 7.12). This genus is involved in the acetoclastic pathway and degrades acetate (Rivière *et al.*, 2009).

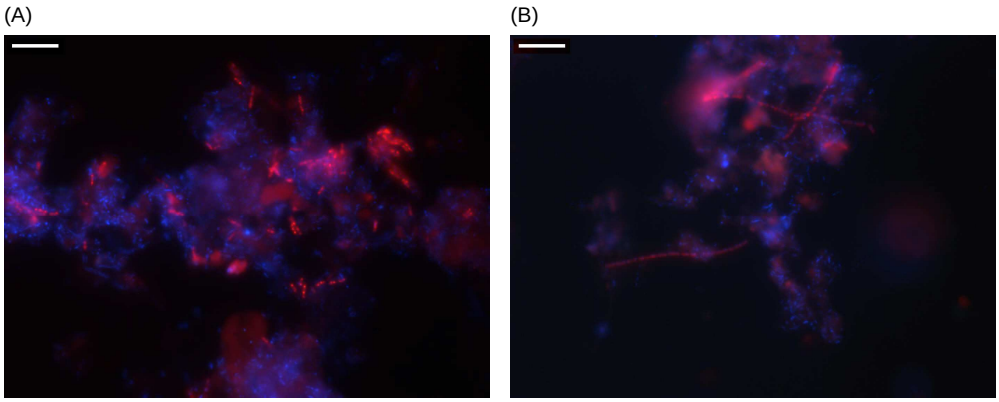


**Figure 7.12.** FISH images of anaerobic sludge with probe MX825 (Cy3) and DAPI in: (A) Stage IB and (B) Stage III. The bar indicates 10 μm.

Biomass samples analysed with the FISH technique indicated the presence of large amounts of microorganisms belonging either to the *Bacteria* or *Archaea* domains. Some of the microorganisms present in anaerobic sludge digesters seem to come from the sludge used as feeding. The thermal pre-treatment of this sludge can promote the disappearance or diminution of some of these microorganisms (like the *Chloroflexi,* observed in this study), which can justify the decrease of the *Bacteria* and *Archaea* domains in Stage II and III. Furthermore during Stage III, apart from the thermal pre-treated AGS, PS was fed, which did not provide a very different composition of the sludge populations with the tested FISH probes in comparison with Stage II (only pre-treated AGS), but caused a decrease in the amount of active organisms in the

anaerobic digester due to the presence of a larger amounts of inorganic solids compared to the AGS biomass.

## 7.5. CONCLUSIONS

The anaerobic digestion of raw AGS, thermal pre-treated AGS and a mixture of thermal pre-treated AGS with raw PS, at SLRs between 0.6 and 1.3 g $VS_{fed}$/L·d, was performed in stable conditions in terms of pH, VFA, TA and free ammonia concentrations, and without inhibitory episodes in a fed-batch CSTR.

The anaerobic BD and solids removal percentage of 44% and 32%, respectively, achieved from the anaerobic digestion of raw AGS were in the range of the values normally reported for WAS.

The thermal pre-treatment of AGS at 133 ºC improved the performance of the anaerobic digester. This enhancement was of 32% and 47% for the BD and solids reduction, respectively.

The mixture of pre-treated AGS with raw PS produced an increase of 17% of solids removal during the anaerobic digestion with respect to the value obtained when only pre-treated AGS was degraded. However the BD decreased from 58% to 53% probably due to the higher $COD_T$/VS ratio of the feeding.

The FISH analysis of biomass samples collected from the anaerobic digester revealed that members of the *Archaea* domain are more abundant than those belonging to the *Bacteria* domain. The total amount of detected microorganism decreased with the change on the feeding from raw AGS, to pre-treated AGS and its mixture with PS, probably due to the disappearance of microorganism during the pre-treatment of the AGS and to the high content of solids different from microorganisms in the PS.

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# Conclusiones generales

Las principales conclusiones de este trabajo, centrado en la aplicación de los sistemas granulares aerobios para el tratamiento de aguas residuales y el posterior tratamiento del lodo granular aerobio generado mediante digestión anaerobia, se describen a continuación:

***Tratamiento del efluente procedente de la industria de productos del mar mediante sistemas granulares aerobios***

El efluente de una industria de productos del mar, sometido previamente a un tratamiento físico-químico, fue tratado con éxito en un sistema granular aerobio operado como un reactor secuencial discontinuo.

La biomasa granular aerobia se formó después de 130 días de la puesta en marcha del reactor. Este largo período de tiempo requerido en comparación con trabajos anteriores puede ser debido a la compleja naturaleza de las aguas residuales industriales. Una vez formados los gránulos aerobios presentaron buenas características de sedimentación: Índice Volumétrico de Lodos (IVL) de 35 mL/g SST y densidad de 60 g $SSV/L_{gránulo}$.

El tratamiento de los efluentes con cargas elevadas y variables, características de la industria de productos del mar, no afectó a la eliminación de materia orgánica. Sin embargo, las propiedades físicas de la biomasa y la eliminación de nitrógeno empeoraron.

El sistema fue capaz de tratar cargas orgánicas entre 2 y 13 kg $DQO_S/m^3 \cdot d$ con una eficacia de eliminación del 90%.

La eliminación de amonio no fue constante a lo largo de todo el período operacional y se vio afectada por el Tiempo de Residencia Celular (TRC), la concentración de amonio libre y la carga de nitrógeno aplicada por superficie de gránulo. Los porcentajes máximos de amonio y nitrógeno total eliminados fueron del 65% y 30%, respectivamente, tratando una carga nitrogenada de 0.45 kg $NH_4^+$-$N/m^3 \cdot d$.

El rendimiento estimado de crecimiento de la biomasa para los gránulos aerobios en el reactor operado a un TRC superior a 4 días fue un 54% menor que el correspondiente a la operación del mismo reactor operado con lodo floculento y a un TRC alrededor de 1 día. Los altos valores del TRC logrados con los sistemas granulares aerobios fueron los responsables de la baja producción de biomasa.

***Efecto del coagulante y floculante en los sistemas de biomasa granular aerobia***

La presencia de cantidades residuales de coagulante y floculante, común en los efluentes industriales pre-tratados, empeoró las características físicas de la biomasa granular aerobia (menor densidad y mayor IVL) y, por tanto, el TRC en el sistema fue menor. La presencia de estos reactivos también disminuyó la tasa de consumo específico de la biomasa.

Las eficacias máximas de eliminación de materia orgánica y nitrógeno alcanzadas fueron similares con y sin reactivos, siendo alrededor del 90% y 60%, respectivamente. Sin embargo, la estabilidad operativa del reactor granular aerobio con la presencia de coagulante-floculante fue peor debido al bajo TRC obtenido.

***Cambios en la distribución del ciclo***

La disminución de la relación hambruna/saciedad desde 10 hasta 5 permitió aumentar las cargas orgánica y nitrogenada tratadas en el sistema en un 33% sin afectar a los rendimientos de eliminación de materia orgánica (97%) y nitrógeno (64%), y con sólo un ligero empeoramiento en las propiedades físicas de la biomasa granular aerobia.

La reducción progresiva de la relación hambruna/saciedad aumentó la capacidad del sistema para acumular compuestos de almacenamiento (PHB) desde 0.052 hasta 0.063 $Cmol_{PHB}/Cmol_{biomasa}$. Esta acumulación benefició el proceso de desnitrificación a lo largo de la fase de hambruna basado en el consumo de estos compuestos de almacenamiento.

La aplicación de una fase anóxica antes de la aerobia e introduciendo la alimentación de una sola vez antes de ésta mejoró la eficacia de eliminación de nitrógeno del purín de cerdo desde un 20% hasta un 60% respecto a la configuración del ciclo con solo fase aerobia. La configuración del ciclo con una fase anóxica y la alimentación simultánea a ésta no solo no aumentó la eficacia de eliminación de nitrógeno, sino que incluso empeoró el proceso de nitrificación.

***Propiedades físicas y estabilidad de la biomasa granular aerobia***

Con los sistemas granulares aerobios operados en modo de reactores secuenciales discontinuos se obtuvieron altas concentraciones de biomasa, alcanzando valores de hasta 8 y 12 g SSV/L empleando como alimentación un medio sintético con acetato y un efluente industrial procedente de una planta de productos del mar, respectivamente.

Los gránulos aerobios desarrollados con ambos tipos de efluentes (sintético e industrial) tuvieron buenas propiedades de sedimentación con valores del IVL de 35-40 mL/g SST y de la densidad entorno a 40-60 g $SSV/L_{gránulo}$.

Se observó que para relaciones F/M inferiores a 1 g DQO/g SSV·d el desarrollo de los gránulos era adecuado, mientras que el aumento de la relación F/M por encima de este valor

provocó un empeoramiento de las características de sedimentación de la biomasa. Concretamente para un valor de la relación F/M de 2.3 g DQO/g SSV·d usando el efluente de la industria de productos del mar los gránulos se rompieron y, consecuentemente, se produjo una pérdida de biomasa del sistema.

La rotura de los gránulos también se pudo atribuir a la presencia de niveles residuales de coagulante y floculante en los efluentes tratados, que pudieron promover el crecimiento rápido del diámetro medio de los gránulos.

***Eliminación de materia orgánica y nitrógeno con los sistemas de biomasa granular aerobia***

Se observó que la eficacia de eliminación de materia orgánica tratando un efluente de una industria de productos del mar y un medio sintético con acetato fue alrededor del 90%, mientras que utilizando purín de cerdo este porcentaje fue del 75%. Lo cual indica que la composición de los efluentes tratados puede influenciar el porcentaje máximo de eliminación de materia orgánica alcanzado, como es el caso de efluentes con alta fracción recalcitrante como el purín de cerdo.

La eliminación de materia orgánica se mantuvo constante a lo largo de toda la operación de los diferentes sistemas granulares aerobios analizados en esta tesis y no se vio afectada por: la aplicación de cargas orgánicas elevadas y variables (de 3 a 13 kg $DQO_S/m^3 \cdot d$), la presencia de reactivos como coagulante y floculante, la reducción de la relación hambruna/saciedad (de 10 a 5) o la aplicación de una fase anóxica antes de la aerobia.

La oxidación de amonio y la eliminación de nitrógeno total fueron más sensibles a los cambios indicados anteriormente y variaron a lo largo de toda la operación de los diferentes sistemas granulares aerobios analizados en esta tesis.

El proceso de nitrificación se llevó a cabo cuando la biomasa granular aerobia predominó dentro del reactor secuencial discontinuo, ya que el TRC alcanzó valores de hasta 4 días. En estas condiciones, el proceso de desnitrificación estuvo limitado por el uso de una fase de reacción completamente aerobia durante la operación del reactor.

***Digestión anaerobia del lodo granular aerobio***

Los experimentos llevados a cabo tanto con ensayos en discontinuo como con la operación de un reactor en continuo indicaron que la digestión anaerobia del lodo granular aerobio tiene un rendimiento similar al referenciado para lodos activos, con un porcentaje de conversión de la materia orgánica en metano entre 30-50%. Por lo que la agregación de la biomasa en gránulos no parece limitar el proceso anaerobio.

Estos resultados obtenidos para el lodo granular aerobio fueron validados con un modelo desarrollado previamente para lodos activos por Mottet *et al*. (2010). Los resultados obtenidos

con este modelo casan bastante bien con los datos experimentales e indican que la BioDegradabilidad (BD) anaerobia de un lodo depende sólo de sus características químicas y no de las propiedades de agregación de la biomasa.

El pre-tratamiento térmico mejoró sensiblemente la digestión anaerobia del lodo granular aerobio cuando su BD inicial fue baja (33%), pero cuando ésta fue alta (49%) la mejora lograda con el pre-tratamiento térmico fue baja, incluso a altas temperaturas, lo que probablemente no justifica la aplicación del pre-tratamiento para este caso.

Los resultados de los ensayos en discontinuo indicaron que una temperatura alrededor de 140 °C podría ser la adecuada para llevar a cabo el pre-tratamiento térmico del lodo granular aerobio procedente de un reactor secuencial discontinuo aerobio tratando purín de cerdo, debido al hecho de que la BD anaerobia mejoró desde el 33% hasta el 60%. La digestión anaerobia en continuo del mismo lodo granular aerobio pre-tratado a 133 ºC, produjo resultados similares con una BD anaerobia alrededor del 58%.

El porcentaje de eliminación de sólidos en el digestor anaerobio alimentado con lodo granular aerobio fue del 32%, aumentando hasta el 47% cuando éste fue pre-tratado térmicamente y hasta el 55% cuando se alimentó la mezcla del lodo granular aerobio pre-tratado y el lodo primario bruto.

El análisis mediante la técnica FISH de las muestras de biomasa recogidas de dentro del digestor anaerobio reveló que los miembros del dominio *Arquea* eran más abundantes que los pertenecientes al dominio *Bacteria*. La cantidad total de microorganismos detectados disminuyó con el cambio en la alimentación desde lodo granular aerobio bruto, hasta lodo granular aerobio pre-tratado y su mezcla con el lodo primario, probablemente debido a la desaparición de los microorganismos durante el pre-tratamiento del lodo granular aerobio y al alto contenido de sólidos diferentes de microorganismos en el lodo primario.

***Algunas reflexiones***

Los sistemas granulares aerobios son una tecnología prometedora, debido a las numerosas ventajas que ofrecen en términos de configuración compacta y de baja producción de lodos con buenas propiedades de sedimentación. La base del proceso es lograr la agregación de la biomasa en gránulos y mantener éstos en condiciones de operación estables. Por tanto la estabilidad de estos agregados es crucial para un funcionamiento adecuado del sistema en términos de calidad del efluente, debido a que la desintegración de los gránulos provoca el aumento de concentración de sólidos en el efluente, así como la pérdida de biomasa y capacidad de tratamiento.

Para evitar la rotura de los gránulos se pueden aplicar purgas selectivas, por ejemplo eliminando los agregados más grandes que son más susceptibles de romper. En otros casos el crecimiento excesivo de los gránulos aerobios se puede limitar impidiendo la entrada de compuestos con la capacidad de favorecer su rápida agregación, como los coagulantes y

floculantes. En este sentido se recomienda un correcto funcionamiento de la unidad de pre-tratamiento físico-químico con el fin de evitar la presencia de altos niveles residuales de estos reactivos.

Respecto a la gestión de los lodos granulares aerobios en esta tesis se ha demostrado la factibilidad de su digestión anaerobia, aunque es necesario más investigación en este campo. Para este propósito es necesario el funcionamiento de más plantas a escala real con la tecnología granular aerobia, puesto que a escala laboratorio el lodo granular producido no es suficiente para llevar a cabo este tipo de experimentos.

# Conclusións xerais

As principais conclusións deste traballo, centrado na aplicación dos sistemas granulares aerobios para o tratamento de augas residuais e o posterior tratamento da lama granular aerobia xerada mediante dixestión anaerobia, descríbense a continuación:

***Tratamento do efluente procedente da industria de produtos do mar mediante sistemas granulares aerobios***

O efluente dunha industria de produtos do mar, sometido previamente a un tratamento físico-químico, foi tratado con éxito nun sistema granular aerobio operado como un reactor secuencial descontinuo.

A biomasa granular aerobia formouse despois de 130 días da posta en marcha do reactor. Este longo período de tempo requirido en comparación con traballos anteriores pode ser debido á complexa natureza das augas residuais industriais. Unha vez formados os gránulos aerobios presentaron boas características de sedimentación: Índice Volumétrico de Lamas (IVL) de 35 mL/g SST e densidade de 60 g $SSV/L_{gránulo}$.

O tratamento dos efluentes con cargas elevadas e variables, características da industria de produtos do mar, non afectou á eliminación de materia orgánica. Non obstante, as propiedades físicas da biomasa e a eliminación de nitróxeno empeoraron.

O sistema foi capaz de tratar cargas orgánicas entre 2 e 13 kg $DQO_S/m^3 \cdot d$ cunha eficacia de eliminación do 90%.

A eliminación de amonio non foi constante ao longo de todo o período operacional e viuse afectada polo Tempo de Residencia Celular (TRC), a concentración de amonio libre e a carga de nitróxeno aplicada por superficie de gránulo. As porcentaxes máximas de amonio e nitróxeno total eliminados foron do 65% e 30%, respectivamente, tratando unha carga nitroxenada de 0.45 kg $NH_4^+$-$N/m^3 \cdot d$.

O rendemento estimado de crecemento da biomasa para os gránulos aerobios no reactor operado a un TRC superior a 4 días foi un 54% menor que o correspondente á operación do mesmo reactor operado con lama floculenta e a un TRC ao redor de 1 día. Os altos valores do TRC acadados cos sistemas granulares aerobios foron os responsables da baixa produción de biomasa.

***Efecto do coagulante e floculante nos sistemas de biomasa granular aerobia***

A presenza de cantidades residuais de coagulante e floculante, común nos efluentes industriais pre-tratados, empeorou as características físicas da biomasa granular aerobia (menor densidade e maior IVL) e, polo tanto, o TRC no sistema foi menor. A presenza destes reactivos tamén diminuíu a taxa de consumo específico da biomasa.

As eficacias máximas de eliminación de materia orgánica e nitróxeno acadadas foron similares con e sen reactivos, sendo arredor do 90% e 60%, respectivamente. Non obstante, a estabilidade operativa do reactor granular aerobio coa presenza de coagulante e floculante foi peor debido ao baixo TRC obtido.

***Cambios na distribución do ciclo***

A diminución da relación fame/saciedade dende 10 ata 5 permitiu aumentar as cargas orgánica e nitroxenada tratadas no sistema sen afectar aos rendementos de eliminación de materia orgánica (97%) e nitróxeno (64%), e con só un lixeiro empeoramento nas propiedades físicas da biomasa granular aerobia.

A redución progresiva da relación fame/saciedade aumentou a capacidade do sistema para acumular compostos de almacenamento (PHB) dende 0.052 ata 0.063 $Cmol_{PHB}/Cmol_{biomasa}$. Esta acumulación beneficiou o proceso de desnitrificación ao longo da fase de fame baseado no consumo destes compostos de almacenamento.

A aplicación dunha fase anóxica antes da aerobia e introducindo a alimentación dunha soa vez antes desta mellorou a eficacia de eliminación de nitróxeno do xurro de porco dende un 20% ata un 60% respecto á configuración do ciclo con só fase aerobia. A configuración do ciclo cunha fase anóxica e a alimentación simultánea a esta non só non aumentou a eficacia de eliminación de nitróxeno, senón que mesmo empeorou o proceso de nitrificación.

***Propiedades físicas e estabilidade da biomasa granular aerobia***

Cos sistemas granulares aerobios operados en modo de reactores secuenciais descontinuos obtivéronse altas concentracións de biomasa, acadando valores de ata 8 e 12 g SSV/L empregando como alimentación un medio sintético con acetato e un efluente industrial procedente dunha planta de produtos do mar, respectivamente.

Os gránulos aerobios desenvolvidos con ámbolos dous tipos de efluentes (sintético e industrial) tiveron boas propiedades de sedimentación con valores do IVL de 35-40 mL/g SST e da densidade entorno a 40-60 g $SSV/L_{gránulo}$.

Observouse que para relacións F/M inferiores a 1 g DQO/g SSV·d o desenvolvemento dos gránulos era adecuado, mentres que o aumento da relación F/M por enriba deste valor provocou un empeoramento das características de sedimentación da biomasa. Concretamente

para un valor da relación F/M de 2.3 g DQO/g SSV·d usando o efluente da industria de produtos do mar os gránulos romperon e, consecuentemente, produciuse unha perda de biomasa do sistema.

A rotura dos gránulos tamén se puido atribuír á presenza de niveis residuais de coagulante e floculante nos efluentes tratados, que puideron promover o crecemento rápido do diámetro medio dos gránulos.

***Eliminación de materia orgánica e nitróxeno cos sistemas de biomasa granular aerobia***

Observouse que a eficacia de eliminación de materia orgánica tratando un efluente dunha industria de produtos do mar e un medio sintético con acetato foi arredor do 90%, mentres que utilizando xurro de porco esta porcentaxe foi do 75%. O cal indica que a composición dos efluentes tratados pode influenciar a porcentaxe máxima de eliminación de materia orgánica acadada, como é o caso de efluentes con alta fracción recalcitrante como o xurro de porco.

A eliminación de materia orgánica mantívose constante ao longo de toda a operación dos diferentes sistemas granulares aerobios analizados nesta tese e non se viu afectada por: a aplicación de cargas orgánicas elevadas e variables (de 3 a 13 kg $DQO_S/m^3 \cdot d$), a presenza de reactivos como coagulante e floculante, a redución da relación fame/saciedade (de 10 a 5) ou a aplicación dunha fase anóxica antes da aerobia.

A oxidación de amonio e a eliminación de nitróxeno total foron máis sensibles aos cambios indicados anteriormente e variaron ao longo de toda a operación dos diferentes sistemas granulares aerobios analizados nesta tese.

O proceso de nitrificación levouse a cabo cando a biomasa granular aerobia predominou dentro do reactor secuencial descontinuo, xa que o TRC acadou valores de ata 4 días. Nestas condicións, o proceso de desnitrificación estivo limitado polo uso dunha fase de reacción completamente aerobia durante a operación do reactor.

***Dixestión anaerobia da lama granular aerobia***

Os experimentos levados a cabo tanto con ensaios en descontinuo como coa operación dun reactor en continuo indicaron que a dixestión anaerobia da lama granular aerobia ten un rendemento similar ao referenciado para lamas activas, cunha porcentaxe de conversión da materia orgánica en metano entre 30-50%. Polo que a agregación da biomasa en gránulos non parece limitar o proceso anaerobio.

Estes resultados obtidos para a lama granular aerobia foron validados cun modelo desenvolvido previamente para lamas activas por Mottet *et al.* (2010). Os resultados obtidos con este modelo casan bastante ben cos datos experimentais e indican que a BioDegradabilidade (BD) anaerobia dunha lama depende só das súas características químicas e non das propiedades de agregación da biomasa.

O pre-tratamento térmico mellorou sensiblemente a dixestión anaerobia da lama granular aerobia cando a súa BD inicial foi baixa (33%), pero cando esta foi alta (49%) a mellora lograda co pre-tratamento térmico foi baixa, mesmo a altas temperaturas, o que probablemente non xustifica a aplicación do pre-tratamento para este caso.

Os resultados dos ensaios en descontinuo indicaron que unha temperatura ao redor de 140 °C podería ser a axeitada para levar a cabo o pre-tratamento térmico da lama granular aerobia procedente dun reactor secuencial descontinuo aerobio tratando xurro de porco, debido ao feito de que a BD anaerobia mellorou dende o 33% ata o 60%. A dixestión anaerobia en continuo da mesma lama granular aerobia pre-tratada a 133º C produciu resultados similares cunha BD anaerobia arredor do 58%.

A porcentaxe de eliminación de sólidos no dixestor anaerobio alimentado con lama granular aerobia foi do 32%, aumentando ata o 47% cando esta foi pre-tratada termicamente e ata o 55% cando se alimentou a mestura da lama granular aerobia pre-tratada e a lama primaria bruta.

A análise mediante a técnica FISH das mostras de biomasa recollidas de dentro do dixestor anaerobio revelou que os membros do dominio *Arquea* eran máis abundantes que os pertencentes ao dominio *Bacteria*. A cantidade total de microorganismos detectados diminuíu co cambio na alimentación dende lama granular aerobia bruta, ata lama granular aerobia pre-tratada e a súa mestura coa lama primaria, probablemente debido á desaparición dos microorganismos durante o pre-tratamento da lama granular aerobia e ao alto contido de sólidos diferentes de microorganismos na lama primaria.

***Algunhas reflexións***

Os sistemas granulares aerobios son unha tecnoloxía prometedora, debido ás numerosas vantaxes que ofrecen en termos de configuración compacta e de baixa produción de lamas con boas propiedades de sedimentación. A base do proceso é lograr a agregación da biomasa en gránulos e manter estes en condicións de operación estables. Polo tanto a estabilidade destes agregados é crucial para un funcionamento axeitado do sistema en termos de calidade do efluente, debido a que a desintegración dos gránulos provoca o aumento de concentración de sólidos no efluente, así como a perda de biomasa e capacidade de tratamento.

Para evitar a rotura dos gránulos pódense aplicar purgas selectivas, por exemplo eliminando os agregados máis grandes que son máis susceptibles de romper. Noutros casos o crecemento excesivo dos gránulos aerobios pódese limitar impedindo a entrada de compostos coa capacidade de favorecer a súa rápida agregación, como os coagulantes e floculantes. Neste sentido recoméndase un correcto funcionamento da unidade de pre-tratamento físico-químico co fin de evitar a presenza de altos niveis residuais destes reactivos.

Respecto á xestión da lama granular aerobia nesta tese demostrouse a factibilidade da súa dixestión anaerobia, aínda que é necesario máis investigación neste campo. Para este

propósito é necesario o funcionamento de máis plantas a escala real coa tecnoloxía granular aerobia, posto que á escala laboratorio a lama granular producida non é suficiente para levar a cabo este tipo de experimentos.

# General conclusions

The main conclusions of this work, which was focused on the application of aerobic granular systems to treat wastewater and the posterior treatment by anaerobic digestion of the generated aerobic granular sludge, are described below:

***Treatment of effluents from the seafood industry in aerobic granular systems***

The effluent from a seafood industry, previously submitted to a physical-chemical treatment, was successfully treated in an aerobic granular system operated as Sequencing Batch Reactor (SBR).

Aerobic granular biomass was considered to be formed 130 days after the reactor start up. This long period required in comparison with previous works, might be due to the complex nature of the industrial wastewater. Once formed the aerobic granules presented good settling characteristics: Sludge Volume Index (SVI) of 35 mL/g TSS and density of 60 g VSS/$L_{granule}$.

The treatment of effluents with high and variable loads, characteristic of seafood industry, did not affect the organic matter removal. However the physical properties of the biomass and the nitrogen removal worsened.

The system was able to treat Organic Loading Rates (OLRs) between 2 and 13 kg $COD_S/m^3 \cdot d$ with an efficiency removal around 90%.

The ammonia removal was not constant along the whole operational period and was affected by the values of the Sludge Retention Time (SRT), the Free Ammonia (FA) concentration and the applied Superficial Nitrogen Loading Rate (SNLR). The maximum percentages of ammonia and Total Nitrogen (TN) removal reached were 65% and 30%, respectively, at a Nitrogen Loading Rate (NLR) of 0.45 kg $NH_4^+$-$N/m^3 \cdot d$.

The biomass growth yield estimated for the aerobic granules operated in a reactor at a SRT higher than 4 days was 54% smaller than the biomass growth yield corresponding to flocculent sludge operated at a SRT around 1 day. The high values of the achieved SRT in the case of the aerobic granular systems were responsible for the low biomass growth yield.

***Effect of coagulant-flocculant reagents on aerobic granular systems***

The presence of trace amounts of coagulant-flocculant reagents, commonly found in pre-treated industrial effluents, worsened the physical characteristics of the granular biomass (lower density, higher SVI) and, therefore, the SRT of the system. The presence of these reagents also decreased the specific consumption rate of the biomass.

The maximum removal efficiencies of organic matter and nitrogen achieved were similar with and without the presence of the reagents, being around 90% and 60%, respectively. However, the operational stability of the aerobic granular reactor with the presence of coagulant-flocculant reagents was worse due to the low SRT obtained.

***Changes on the cycle distribution***

A decrease of the famine/feast ratio from 10 to 5 allowed increasing the treated OLR and NLR without affecting the removal efficiencies of organic matter (97%) and nitrogen (64%) and with only a slight detriment on the physical properties of aerobic granular biomass.

The progressive reduction of the famine/feast ratio increased the capacity of the system to accumulate storage compounds (PHB) from 0.052 to 0.063 $Cmol_{PHB}/Cmol_{biomass}$. This accumulation benefitted the occurrence of the denitrification process along the famine phase based on the consumption of these storage compounds.

The implementation of an anoxic phase before the aerobic one with a pulse-fed mode improved the nitrogen removal efficiency of the pig manure from 20 to 60% respect to the cycle configuration with a complete aerobic phase. The cycle configuration with an anoxic phase and simultaneous feeding did not enhance the nitrogen removal efficiency and even worsened the nitrification process.

***Physical properties and stability of aerobic granular biomass***

The aerobic granular systems operated in SBR mode allowed obtaining high biomass concentrations inside the reactors, reaching values as high as 8 and 12 g VSS/L fed with a synthetic medium containing acetate or with an industrial effluent from the seafood industry, respectively.

The aerobic granules developed with both kinds of effluents (synthetic and industrial) exhibited good settling properties with values of SVI around 35-40 mL/g TSS and densities of 40-60 g $VSS/L_{granule}$.

Values of the F/M ratio lower than 1 g $COD_s$/g VSS·d were suitable for a good development of aerobic granules, while the increase of the F/M ratio above this value provoked a worsening in the biomass settling characteristics. In this work a value of the F/M ratio of 2.3 g

$COD_S$/g VSS·d using the effluent from seafood industry provoked the granules breakage and biomass loss from the system.

Breakage of the granules can also be attributed to the presence of residual levels of coagulant-flocculant reagents in the treated effluents, which can promote the fast growth of the granules average diameter.

***Organic matter and nitrogen removals with aerobic granular systems***

From the results obtained it was observed that the organic matter removal efficiency treating an effluent from a seafood industry and a synthetic medium containing acetate was around 90%, while when pig manure was used this percentage was of 75%. This fact can indicate that the composition of the treated effluent influences the maximum achievable COD removal percentages as it is the case of effluents with high recalcitrant fraction like the pig manure.

The organic matter removal remained constant along the whole operation of the different aerobic granular systems assayed in this thesis and it was not affected by: the application of high and variable OLRs (from 3 to 13 kg $COD_S/m^3$·d), the presence of coagulant-flocculant reagents, the reduction of the famine/feast ratio (from 10 to 5) or the implementation of an anoxic phase previous to the aerobic one.

The ammonia oxidation and total nitrogen removal efficiencies were more sensitive to the previous indicated changes and they varied along the whole operation of the different aerobic granular systems assayed in this thesis.

The nitrification process took place when the aerobic granular biomass predominated inside the SBRs, since the SRT reached values up to 4 days. In these conditions the denitrification process was limited by the use of a complete aerobic reaction phase during the performance of the SBR.

***Anaerobic digestion of Aerobic Granular Sludge***

The experiments carried out in batch tests and in a continuous fed-batch anaerobic digester indicated that the anaerobic digestion of Aerobic Granular Sludge (AGS) has a similar performance than that reported for Waste Activated Sludge (WAS), with a conversion of the organic matter into methane between 30-50%. The aggregation of the biomass into granules does not seem to limit the anaerobic process.

These results obtained for the AGS were validated with a previously developed model for WAS by Mottet *et al.* (2010). The results obtained with this model fitted quite well with the experimental data which indicated that the anaerobic BioDegradability (BD) of a sludge depended only on its chemical characteristics and not on the aggregation properties of the biomass.

A thermal pre-treatment was proposed to enhance the anaerobic digestion of the AGS when its initial BD was low (33%). If the BD of the untreated AGS was high (49%) the enhancement achieved with the thermal pre-treatment was low even at high temperatures, which probably does not justify the application of this pre-treatment.

The results from the batch tests indicated that a temperature around 140 ºC could be adequate to carry out the thermal pre-treatment of the AGS coming from an aerobic granular SBR treating pig manure, due to the fact that the anaerobic BD was enhanced from 33% to 60%. The continuous anaerobic digestion of the same AGS pre-treated at 133 ºC gave similar results with an anaerobic BD around 58%.

The solids removal percentage in the anaerobic digester fed with non pre-treated AGS was 32%, increasing to 47% with thermal pre-treated AGS and to 55% with a mixture of thermal pre-treated AGS and raw Primary Sludge (PS).

The FISH analysis of biomass samples collected from the anaerobic digester revealed that members of the *Archaea* domain were more abundant than those belonging to the *Bacteria* domain. The total amount of detected microorganisms decreased with the change on the feeding from raw AGS, to pre-treated AGS and its mixture with PS, probably due to the disappearance of microorganisms during the pre-treatment of the AGS and to the high content of solids different from microorganisms in the PS.

***Some reflexions***

The aerobic granular systems are a promising technology due to the numerous advantages that offer in terms of compact configuration and low sludge production with good settling properties. The basis of the process is to achieve the aggregation of the biomass in granules and to maintain them in stable operational conditions. Therefore the stability of these aggregates is crucial to obtain a successful operation in terms of effluent quality, because the disintegration of the granules provokes the increase of solids concentration in the effluent as well as the loss of biomass retention and treatment capacity.

To avoid episodes of granules breakage selective purges can be applied, for example removing the bigger aggregates that are most susceptible to break. In another cases the excessive growth of the aerobic granules can be limited by preventing the inlet of compounds with the capacity to favour the faster aggregation, like the coagulant-flocculant reagents. In this sense a correct operation of the physical-chemical pre-treatment process is recommended in order to avoid the presence of high residual levels of coagulant-flocculant reagents.

Respect to the management of the aerobic granular sludge in this thesis the feasibility of its anaerobic digestion was demonstrated, although more investigation in this field is necessary. For this purpose the operation of full scale plants with the aerobic granular technology are necessary, since at laboratory scale the sludge produced is not enough to carry out this type of experiments.

# List of acronyms and symbols

## Acronyms

| | |
|---|---|
| **AGS** | Aerobic Granular Sludge |
| **APHA** | American Public Health Association |
| **AUSB** | Aerobic Upflow Sludge Blanket |
| **AWWA** | American Water Works Association |
| **BSA** | Bovine Serum Albumine |
| **C** | Cytosine |
| **CAS** | Conventional Activated Sludge |
| **CF** | Coagulant-Flocculant |
| **COD/N** | Chemical oxygen demand to nitrogen ratio |
| **CSTR** | Continuous Stirring Tank Reactor |
| **Cy3** | Cyanine 3 |
| **Cy5** | Cyanine 5 |
| **DAPI** | 4',6-DiAmidino-2-Phenylindole |
| **DNA** | Deoxyribo-Nucleic Acid |
| **EC** | European Commission |
| **EDC** | Endocrine Disrupter Compound |
| **EDTA** | Ethylene-Diamine-Tetra-Acetic acid |
| **EPS** | Extracellular Polymeric Substances |
| **EU** | European Union |
| **F** | Formamide |
| **FAS** | Ferrous Ammonium Sulphate |
| **FISH** | Fluorescent *In Situ* Hybridization |
| **FITC** | Fluorescein IsoThioCyanate |
| **G** | Guanine |
| **GC** | Gas Cromatography |
| **GSBR** | Granular Sequencing Batch Reactor |
| **IWA** | International water Association |

| | |
|---|---|
| **MBR** | Membrane Biological Reactor |
| **MF/UF** | Micro- or Ultra- Filtration |
| **MLSS** | Mixed Liquor Suspended Solids |
| **N** | Nitrogen |
| **$NO_X^-$** | Nitrogen oxides |
| **P** | Phosphorus |
| **p.e.** | Population equivalents |
| **PBS** | Phosphate Buffer Solution |
| **PH2MV** | Poly-Hydroxy-2-MethylValerate |
| **PHA** | Poly-Hydroxy-Alkanoate |
| **PHB** | Poly-Hydroxy-Butyrate |
| **PHV** | Poly-Hydroxy-Valerate |
| **PLC** | Programmable Logic Controller |
| **PS** | Primary Sludge |
| **rRNA** | Ribosomal Ribo-Nucleic Acid |
| **SBBGR** | Sequencing Batch Biofilm Granular Reactor |
| **SBR** | Sequencing Batch Reactor |
| **SEM** | Scanning Electron Microscope |
| **T** | Thymine |
| **Tris** | Tris(hydroxymethyl)aminomethane |
| **UASB** | Upflow Anaerobic Sludge Blanket |
| **WAS** | Waste Activated Sludge |
| **WWTP** | WasteWater Treatment Plant |

## Symbols

| | | |
|---|---|---|
| **BD** | BioDegradability | % |
| **BMP** | BioMethane Potential | N-$mL_{CH4}$/g COD |
| **COD** | Chemical Oxygen Demand | g/L |
| **COD/N** | Chemical oxygen demand to nitrogen ratio | -- |
| **DO** | Dissolved Oxygen | mg $O_2$/L |
| **F/M** | Food to Microorganism ratio | -- |
| **FA** | Free Ammonia | g N/L |
| **H/D** | Height to Diameter ratio | -- |
| **HRT** | Hydraulic Retention Time | d |
| **IA** | Intermediate Alkalinity | g $CaCO_3$/L |
| **IC** | Inorganic Carbon | g/L |

| | | |
|---|---|---|
| **IN** | Inorganic Nitrogen | g/L |
| **$k_La$** | Gas-liquid oxygen transfer coefficient | $d^{-1}$ |
| **NLR** | Nitrogen Loading Rate | kg N/($m^3$·d) |
| **OLR** | Organic Loading Rate | kg COD/($m^3$·d) |
| **PA** | Partial Alkalinity | g $CaCO_3$/L |
| **PMA** | Potential Methanogenic Activity | g COD-$CH_4$/(g $VS_{reactor}$·d) |
| **SNLR** | Superficial Nitrogen Loading Rate | kg N/($m^2$·d) |
| **SOUR** | Specific Oxygen Uptake Rate | g $O_2$/(g VSS·d) |
| **SRT** | Solid Retention Time | d |
| **SVI** | Sludge Volume Index | mL/g TSS |
| **TA** | Total Alkalinity | g $CaCO_3$/L |
| **TC** | Total Carbon | g/L |
| **TKN** | Total Kjeldhal Nitrogen | g/L |
| **TN** | Total Nitrogen | g/L |
| **TOC** | Total Organic Carbon | g/L |
| **TS** | Total Solids | g/L |
| **TSS** | Total Suspended Solids | g/L |
| **VFA** | Volatile Fatty Acid | g/L |
| **VS** | Volatile Solids | g/L |
| **VSS** | Volatile Suspended Solids | g/L |
| **Y** | Yield coefficient | g VSS/g COD |

# List of publications

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