UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Ingeniería Química

Autotrophic nitrogen removal in granular sequencing batch reactors

Memoria presentada por
José Ramón Vázquez Padín
Para optar al grado de Doctor por la
Universidad de Santiago de Compostela

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Informan:

Que la memoria titulada “Autotrophic nitrogen removal in granular sequencing batch reactors”, que para optar al grado de Doctor de Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Don José Ramón Vázquez Padín, ha sido realizada bajo nuestra inmediata dirección en el Departamento de Ingeniería Química de la Universidad de Santiago de Compostela.

Y para que así conste, firman el presente informe en Santiago de Compostela, 24 de junio de 2009.

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"*No entiendes realmente algo a menos que seas capaz de explicárselo a tu abuela*"

Albert Einstein

"El azar no existe; Dios no juega a los dados"

Albert Einstein

"Dios no sólo juega a los dados: a veces los tira donde no se pueden ver"

Stephen Hawking

"*You do not really understand something unless you can explain it to your grandmother*"

Albert Einstein

"God does not play dice"

Albert Einstein

"Not only does God play dice, but he sometimes throws them where they cannot be seen"

Stephen Hawking

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Table of contents

Objetivos y resumen

Esta tesis se encuadra en el marco de la depuración de aguas y más concretamente en la eliminación de nitrógeno de las aguas residuales. La eliminación de nutrientes (principalmente N y P) en las aguas residuales es necesaria para evitar la proliferación de algas o eutrofización de las aguas receptoras. Los procesos biológicos son los más utilizados para llevar a cabo la eliminación de nutrientes debido a su menor coste en comparación con los físico-químicos. El proceso típico de eliminación de nitrógeno consiste en la oxidación de amonio a nitrato, en dos pasos: 1) oxidación de amonio a nitrito por medio de bacterias oxidantes de amonio (BOA) y 2) oxidación de nitrito a nitrato por medio de bacterias oxidantes de nitrito (BON). Una vez el amonio se ha oxidado a nitrato, éste es reducido a nitrógeno gas por bacterias heterótrofas en ausencia de oxígeno. Sin embargo, este proceso no es el más adecuado para tratar aguas con alta carga nitrogenada, es decir aguas con baja relación DQO/N. En este caso se debe añadir una fuente externa de carbono que encarece el proceso. Una alternativa para eliminar nitrógeno de estos efluentes, surgida en los últimos años, es la eliminación autótrofa de nitrógeno llevada a cabo por bacterias Anammox que combinan amonio y nitrito para dar nitrógeno gas y una pequeña cantidad de nitrato en condiciones anaerobias. Este proceso resultó de gran interés para tratar aguas de salida de digestores anaerobios ya que éstas contienen altas concentraciones de nitrógeno y poca materia orgánica biodegradable. Sin embargo, el lento crecimiento de las bacterias autótrofas, en comparación con las heterótrofas dificulta la puesta en marcha y posterior desarrollo del proceso. Dos configuraciones alternativas son posibles para llevar a cabo el proceso de eliminación autótrofa de nitrógeno: 1) proceso en dos etapas: nitrificación parcial del 50% del amonio a nitrito en el primer reactor que alimentaría un posterior reactor Anammox, 2) ambos procesos llevados a cabo en un único reactor, proceso conocido como CANON ("Complete Autotrophic Nitrogen Removal Over Nitrite").

La eliminación autótrofa de nitrógeno de la corriente de salida de los digestores anaerobios en las plantas de tratamientos de aguas residuales presenta una serie de ventajas entre las que se encuentran:

- El aumento de la capacidad de eliminación de nitrógeno de la planta.
- La reducción de la cantidad de lodos producidos ya que la productividad de la biomasa autótrofa (nitrificante y Anammox) es menor que la heterótrofa (desnitrificante).
- La producción de inferiores cantidades de gases de efecto invernadero (N_2O y NO).
- Un aumento del aprovechamiento energético en el digestor anaerobio ya que al eliminar el nitrógeno de la corriente de rechazo se puede aumentar el tiempo de residencia hidráulico en los decantadores primarios de las EDAR. Esto reduce los requerimientos energéticos para aireación manteniendo la eficacia de eliminación de nitrógeno en la planta.

Los procesos biológicos pueden llevarse a cabo en sistemas con biomasa en suspensión o en forma de biopelículas (biomasa adherida o granular). El uso de sistemas basados en biopelículas permite el tratamiento de mayores cargas en menores volúmenes de reactor y, por lo tanto, está recomendado para el tratamiento de efluentes con elevadas cargas. Además el hecho de que la biomasa se desarrolle de esta forma permite retener mayores cantidades, lo cual es beneficioso en el caso de utilizar microorganismos de lento crecimiento como es el caso de las bacterias nitrificantes y Anammox.

En los años 90 se desarrolló en los Países Bajos una tecnología basada en la formación de biomasa granular en condiciones aerobias. Esta tecnología se fundamenta en el desarrollo de biopelículas sin necesidad de material de soporte. Los primeros trabajos se desarrollaron estudiando la autoagregación de bacterias heterótrofas alimentadas con una fuente de carbono, formando agregados cuya principal característica es su elevada velocidad de sedimentación. Para ello, se utiliza un reactor operado de forma discontínua (SBR: Sequencing Batch Reactor) con una elevada relación altura/diámetro y se somete la biomasa a periodos cíclicos de saciedad y hambruna. El empleo de sistemas granulares presenta las siguientes ventajas:

- Permiten alcanzar mayores concentraciones de biomasa en el reactor, disminuyendo la productividad de los lodos y aumentando la capacidad del reactor.
- Se minimiza el lavado de biomasa, hecho fundamental al trabajar con bacterias de lento crecimiento.
- La velocidad de decantación de la biomasa es mucho mayor que la de los lodos activos, disminuyendo la superficie necesaria para el decantador (se puede llegar incluso a no necesitar decantador).

En base a todo lo anteriormente citado, en la presente tesis se han estudiado las condiciones de operación adecuadas para la autoagregación de poblaciones de lento crecimiento como las nitrificantes (BOA y BON) y las Anammox, cada una de ellas por separado (Capítulos 3, 4 y 5).

Posteriormente, se han combinado poblaciones de BOA y Anammox en una única unidad para llevar a cabo el proceso CANON, este proceso ha sido desarrollado a temperatura ambiente en dos tipos de reactores (Capítulo 6). A continuación, con el fin de analizar la operación de los gránulos a nivel microscópico se llevó a cabo un estudio por medio de microelectrodos para el seguimiento de perfiles de concentración en el interior de dichos gránulos (Capítulo 7).

Con los datos experimentales obtenidos en un trabajo previo en este tipo de sistemas, se ha desarrollado un modelo que ha permitido simular el comportamiento de gránulos aerobios en los que se llevaba a cabo la eliminación de nitrógeno a través del proceso convencional de nitrificación-desnitrificación (Capítulo 8).

A continuación se detallarán los contenidos principales de cada uno de los capítulos de la presente tesis.

En el capítulo 1, se presenta una revisión bibliográfica actualizada de los estudios realizados hasta la fecha sobre la eliminación de nitrógeno en las aguas residuales haciendo especial hincapié en la eliminación autótrofa de amonio (proceso Anammox). Se presenta también información relativa a los sistemas de granulación aerobia, la microbiología de los procesos implicados y el estado del arte del modelado de estos sistemas.

En el capítulo 2, se desarrollan los materiales y métodos utilizados a lo largo de los experimentos realizados en los capítulos posteriores.

En el capítulo 3, se parte de gránulos meramente nitrificantes operando a temperatura ambiente (18−24 ºC). En un trabajo previo a esta tesis, dichos gránulos habían sido desarrollados con acetato como fuente de carbono y se les había retirado progresivamente la fuente de carbono de la alimentación. La carga

de amonio aplicada al inicio de la experimentación era de 0,4 g N L^{-1} d^{-1}. Al analizar la evolución de las especies nitrogenadas durante el ciclo se observaron pequeñas acumulaciones de nitrito durante el mismo que indicaron que las velocidades de las dos etapas de la nitrificación (oxidación de amonio y oxidación de nitrito) se desacoplaban al inicio del ciclo, es decir, cuando la concentración de amonio era máxima. La carga máxima que se consiguió nitrificar a nitrato fue de 0,4 g N L^{-1} d^{-1}. Al doblar la carga nitrogenada aplicada, se observó una acumulación permanente de nitrito dejando patente una limitación en la velocidad de crecimiento de las BON a pesar de trabajar con una concentración de oxígeno disuelto cercana a la de saturación. Con el objetivo de evitar altas concentraciones de amonio que pudieran ser inhibitorias, se pasó a alimentar el reactor durante todo el periodo aireado del ciclo. De la estimación de las velocidades de las distintas etapas de la reacción, se determinó que la transferencia de oxígeno desde la fase líquida al interior del gránulo constituía la etapa limitante. Dado que las BOA tienen mayor afinidad por el oxígeno que las BON, estas últimas se vieron más afectadas por la limitación de oxígeno, acumulándose nitrito en el sistema. Una vez descubierto el potencial de los gránulos nitrificantes para producir nitrito, se fijó como objetivo la obtención de la nitrificación parcial, lo que implica la oxidación del 50% del amonio a nitrito evitando la oxidación a nitrato. La nitrificación parcial se consiguió disminuyendo la concentración de oxígeno disuelto en el medio líquido. En estas condiciones, la máxima carga de nitrógeno parcialmente oxidada fue de 1 g N L^{-1} d^{-1} con una concentración de oxígeno disuelto en el rango 2,0—3,5 mg O_2 L^{-1}. Los gránulos mantuvieron su diámetro medio (en torno a 3 mm), su integridad y sus buenas características de sedimentación, con un bajo índice volumétrico y una alta velocidad de sedimentación (100 m h^{-1}) durante la operación del reactor.

En el capítulo 4 se estudia la granulación de BON con el fin de corroborar que la acumulación de nitrito se debía a la competición por oxígeno y no a que las bacterias oxidantes de nitrito presentan más dificultades para crecer formando agregados que las oxidantes de amonio. El concepto granulación aerobia se asocia generalmente a bacterias heterótrofas, sin embargo se observó en el capítulo 4 al igual que en el 3 que las bacterias autótrofas también pueden desarrollarse formando agregados o gránulos sin necesidad de ser sometidas a periodos de saciedad y hambruna. Dado que las BON resultan indeseables en un reactor CANON, pues compiten con las bacterias Anammox por el nitrito, se ha de poner especial atención para evitar su desarrollo. El objetivo de este capítulo fue el estudio de la evolución de la estructura de los gránulos y de las poblaciones bacterianas al cambiar la fuente de nitrógeno alimentada de amonio a nitrito. Los gránulos, cuyo tamaño era de 0,8 mm, estaban colonizados por *Nitrosomonas* (oxidantes de amonio) y *Nitrospira* (oxidantes de nitrito). Las bacterias del género *Nitrospira* se caracterizan por ser estrategas de la k, es decir, bacterias que tienen mayor afinidad por el sustrato pero menor velocidad máxima de crecimiento que las estrategas de la r (*Nitrobacter* en el caso de las NOB). Una vez cambiada la fuente de nitrógeno alimentada de amonio a nitrito y tras 250 días de operación no se observó un deterioro de la biomasa granular y la población de BON mayoritaria en este reactor resultó ser la *Nitrobacter*.

En el capítulo 5 se estudia el proceso de granulación de bacterias Anammox en el tratamiento de un efluente sintético salino simulando efluentes industriales, como por ejemplo las aguas residuales generadas en la industria conservera de pescado. La presencia de concentraciones de sal (NaCl) de hasta 10 g L^{-1} no sólo no producía efectos negativos significativos sobre la velocidad de reacción de estos microorganismos sino que fomentaba las interacciones entre agregados favoreciendo la granulación. El diámetro medio de los gránulos aumentó un 24% y la concentración de biomasa aumentó un 60% después de añadir NaCl, en una primera etapa se añadieron 5 g NaCl L^{-1} en la alimentación y en la segunda etapa se añadieron 10 g NaCl L^{-1}, proceso que tuvo una duración total de 54 días. Las actividades máximas específicas registradas fueron de 0,45 g N (g VSS)$^{-1}$ d^{-1}.

En el capítulo 6, se estudia el desarrollo del proceso CANON en dos sistemas diferentes: una columna de borboteo con aireación continua y otra con aireación pulsada. Ambas unidades se pusieron en marcha como reactores nitrificantes en los que se establecieron las condiciones de operación adecuadas para llevar a cabo la oxidación parcial de amonio en un 50% a una temperatura de alrededor de 20 ºC.

En el reactor con aireación continua la biomasa Anammox se desarrolló en el interior de los gránulos nitrificantes en los que se llevaba a cabo el proceso de nitrificación parcial. A pesar de las condiciones adversas presentes en el medio líquido: alta concentración de oxígeno disuelto y de nitrito, la elevada actividad oxidante de amonio en las capas externas de los gránulos elimina por completo el oxígeno en el interior de los mismos creando una zona anóxica rica en amonio y nitrito, propicia para el desarrollo de biomasa Anammox (proceso CANON). En esta unidad la máxima carga tratada fue de 1,1 g N L^{-1} d^{-1} cuando el tamaño de los gránulos era de 3,2 mm.

En el reactor con aireación pulsada se estudió el arranque de un reactor CANON minimizando el consumo de aire para el aporte del oxígeno requerido. El oxígeno a este reactor se aportó mediante aire pulsado con una frecuencia de 0.09 s^{-1}. Una vez desarrollados los gránulos nitrificantes con un diámetro de 1,6 mm que llevaban a cabo la nitrificación parcial se inoculó el reactor con biomasa poco enriquecida en bacterias Anammox. En esta unidad la biomasa Anammox se mantuvo en forma de agregados mientras que la población de BOA permaneció fundamentalmente en forma de flóculos aunque una pequeña parte se encontraba en las capas más externas de los gránulos Anammox. El arranque del proceso CANON fue muy rápido y en 35 días la carga de nitrógeno eliminada fue de 0,25 g N L^{-1} d^{-1}, siendo la máxima carga tratada de 0,45 g N L^{-1} d^{-1} con una concentración de oxígeno de 0,3—0,5 mg O_2 L^{-1}.

En el capítulo 7, se llevó a cabo un estudio microscópico de los gránulos del reactor CANON con aireación continua utilizando microelectrodos para medir la concentración de oxígeno disuelto y de nitrito (los sustratos limitantes en el proceso CANON). Esta herramienta se combinó con la técnica FISH de identificación de poblaciones bacterianas. Los resultados indicaron una clara estratificación de poblaciones en la profundidad de los gránulos con BOA concentradas en una capa externa de 400 μm, mientras que las bacterias Anammox estaban situadas entre las 400 y las 1000 μm de profundidad. Los resultados de la determinación de los perfiles de concentración de oxígeno disuelto en los gránulos, en experimentos llevados a cabo a diferentes concentraciones de éste en el medio líquido, indicaron que el flujo de oxígeno hacia el interior de los gránulos aumentaba al aumentar la concentración de oxígeno disuelto en el medio. Esto demuestra que la actividad oxidante de amonio está limitada por la concentración de oxígeno disuelto a pesar que el reactor se operó en condiciones cercanas al 100% de saturación con aire. Del mismo modo, los perfiles de concentración de nitrito realizados variando la concentración de éste en el medio líquido corroboraron que una concentración cercana a los 9 mg N L^{-1} es necesaria para que la biomasa Anammox no esté limitada por nitrito. Con la información obtenida en este trabajo se infiere que existen unos valores óptimos de concentración de amonio y concentración de nitrito en el medio líquido que permiten optimizar la estrategia de control y la operación del proceso CANON.

En el capítulo 8, se desarrolló un modelo capaz de simular para un reactor granular aerobio tanto las concentraciones de nutrientes en el medio líquido como las propiedades físicas de los gránulos formados, en términos de sólidos en suspensión volátiles y densidad de los mismos. Para conseguirlo, se utilizó un modelo en una dimensión empleando el software Aquasim y basado en el ASM3 modificado porque se describió la nitrificación como un proceso en dos etapas. Con respecto a los demás modelos ya publicados en este tema, se incluyeron modificaciones necesarias para simular adecuadamente las concentraciones y densidad de

biomasa que fueron la introducción de un perfil de porosidad variable en la profundidad del gránulo y la asignación de valores de densidad distintos para bacterias heterótrofas (350 g DQO $(L_{gránulo})^{-1}$) y autótrofas (150 g DQO $(L_{gránulo})^{-1}$). Se definieron en el modelo cinco tipos de sustancias sólidas: bacterias heterótrofas, bacterias oxidantes de amonio y oxidantes de nitrito, polímeros de almacenamiento y materia inerte y seis tipos de sustrato disueltos en el medio liquido: oxígeno, DQO, alcalinidad, amonio, nitrito y nitrato.

Con los trabajos realizados en esta tesis se ha conseguido información valiosa para la posible aplicación del proceso autótrofo CANON para eliminar el nitrógeno de efluentes a temperatura ambiente.

Obxectivos e resumo

Esta tese encádrase no marco da depuración de augas e máis concretamente na eliminación de nitróxeno das augas residuais. A eliminación de nutrientes (principalmente N e P) nas augas residuais é necesaria para evitar a proliferación de algas ou eutrofización das augas receptoras. Os procesos biolóxicos son os máis utilizados para levar a cabo a eliminación de nutrientes debido ao seu menor custo en comparación cos fisicoquímicos. O proceso típico de eliminación de nitróxeno consiste na oxidación de amonio a nitrato, en dous pasos: 1) oxidación de amonio a nitrito por medio de bacterias oxidantes de amonio (BOA) e 2) oxidación de nitrito a nitrato por medio de bacterias oxidantes de nitrito (BON). Unha vez o amonio oxidado ata nitrato, este é reducido a nitróxeno gas por bacterias heterótrofas en ausencia de osíxeno. Con todo, este proceso non é o máis axeitado para tratar augas con alta carga nitroxenada, é dicir augas con baixa relación DQO/N. Neste caso débese engadir unha fonte externa de carbono que encarece o proceso. Unha alternativa para eliminar nitróxeno destes efluentes, xurdida nos últimos anos, é a eliminación autótrofa de nitróxeno levada a cabo por bacterias Anammox que combinan amonio e nitrito para dar nitróxeno gas e unha pequena cantidade de nitrato en condicións anaerobias. Este proceso resultou interesante para tratar augas de saída de dixestores anaerobios xa que estas conteñen altas concentracións de nitróxeno e pouca materia orgánica biodegradable. Con todo, o lento crecemento das bacterias autótrofas, en comparación coas heterótrofas dificulta a posta en marcha e posterior desenvolvemento do proceso. Dúas configuracións alternativas son posibles para levar a cabo o proceso de eliminación autótrofa de nitróxeno: 1) proceso en dúas etapas: nitrificación parcial do 50% do amonio a nitrito no primeiro reactor que alimentaría un posterior reactor Anammox, 2) ambos procesos levados a cabo nun único reactor, proceso coñecido como CANON ("Complete Autotrophic Nitrogen Removal Over Nitrite").

A eliminación autótrofa de nitróxeno da corrente de saída dos dixestores anaerobios nas plantas de tratamentos de augas residuais presenta unha serie de vantaxes entre as que se atopan:

- O aumento da capacidade de eliminación de nitróxeno da planta.
- A redución da cantidade de lodos producidos xa que a produtividade da biomasa autótrofa (nitrificante e Anammox) é menor que a heterótrofa (desnitrificante).
- A produción de inferiores cantidades de gases de efecto invernadoiro (N_2O e NO).
- Un aumento do aproveitamento enerxético no dixestor anaerobio xa que ao eliminar o nitróxeno da corrente de rexeitamento pódese aumentar o tempo de residencia hidráulico nos decantadores primarios das EDAR. Isto diminue a necesidade de aireación mantendo a eficacia de eliminación de nitróxeno da planta.

Os procesos biolóxicos pódense levar a cabo en sistemas con biomasa en suspensión ou en forma de biopelículas (biomasa adherida ou granular). O uso de sistemas baseados en biopelículas permite o tratamento de maiores cargas en menores volumes de reactor e, xa que logo, está recomendado para o tratamento de efluentes con elevadas cargas. Ademais o feito de que a biomasa creza desta forma permite reter maiores cantidades da mesma, o cal é beneficioso no caso de utilizar microorganismos de lento crecemento como é o caso das bacterias nitrificantes e Anammox.

Nos anos 90 desenvolveuse nos Países Baixos unha tecnoloxía baseada na formación de biomasa granular en condicións aerobias. Esta tecnoloxía fundaméntase no desenvolvemento de biopelículas sen necesidade de material de soporte. Os primeiros traballos desenvolvéronse estudando a autoagregación de bacterias heterótrofas alimentadas cunha fonte de carbono, formando agregados cuxa principal característica era a súa elevada velocidade de sedimentación. Para iso, utilízase un reactor operado de forma descontinua (SBR: Sequencing Batch Reactor) cunha elevada relación altura/diámetro e sométese a biomasa a períodos cíclicos de saciedade e fame. O emprego de sistemas granulares presenta as seguintes vantaxes:

- Permiten alcanzar maiores concentracións de biomasa no reactor, diminuíndo a produtividade dos lodos e aumentando a capacidade do reactor.
- Minimízase o lavado de biomasa, feito fundamental ao traballar con bacterias de lento crecemento.
- A velocidade de decantación da biomasa é moito maior que a dos lodos activos, diminuíndo a superficie necesaria para o decantador (pódese chegar inclusive a non necesitar decantador).

En base a todo o anteriormente citado, na presente tese estudáronse as condicións de operación axeitadas para a autoagregación de poboacións de lento crecemento como as nitrificantes (BOA e BON) e as Anammox, cada unha delas por separado (Capítulos 3, 4 e 5).

Posteriormente, combináronse poboacións de BOA e Anammox nunha única unidade para levar a cabo o proceso CANON, este proceso foi desenvolvido a temperatura ambiente en dous tipos de reactores (Capítulo 6). A continuación, co fin de analizar a operación dos gránulos a nivel microscópico levouse a cabo un estudo por medio de microelectrodos para o seguimento de perfís de concentración no interior de devanditos gránulos (Capítulo 7).

Cos datos experimentais obtidos nun traballo previo neste tipo de sistemas, desenvolveuse un modelo que permitiu simular o comportamento de gránulos aerobios nos que se levaba a cabo a eliminación de nitróxeno a través do proceso convencional de nitrificación-desnitrificación (Capítulo 8).

A continuación, detallaranse os contidos principais de cada un dos capítulos da presente tese.

No capítulo 1, preséntase unha revisión bibliográfica actualizada dos estudos realizados ata a data sobre a eliminación de nitróxeno nas augas residuais facendo especial fincapé na eliminación autótrofa de amonio (proceso Anammox). Preséntase tamén información relativa aos sistemas de granulación aerobia, a microbioloxía dos procesos implicados e o estado da arte do modelado destes sistemas.

No capítulo 2, desenvólvense os materiais e métodos utilizados ao longo dos experimentos realizados nos capítulos posteriores.

No capítulo 3 pártese de gránulos nitrificantes operando a temperatura ambiente (18—24 ºC) que foran previamente desenvolvidos con acetato como fonte de carbono e aos que se lles retirou progresivamente dita fonte de carbono da alimentación. A carga de amonio aplicada ao comezo da experimentación era de 0,4 g N L^{-1} d^{-1}. Ao analizar a evolución das especies nitroxenadas durante o ciclo observáronse pequenas acumulacións de nitrito durante o mesmo que indicaron que as velocidades das dúas etapas da nitrificación (oxidación de amonio e oxidación de nitrito) eran diferentes ao comezo do ciclo, é dicir, cando a concentración de amonio era máxima. A carga máxima que se conseguiu nitrificar a nitrato foi de 0,4 g N L^{-1} d^{-1}. Ao dobrar a carga nitroxenada aplicada, observouse unha acumulación permanente de nitrito deixando patente unha limitación na velocidade de crecemento das BON malia traballar cunha concentración de osíxeno disolto

próxima á de saturación. Co obxectivo de evitar altas concentracións de amonio que puidesen ser inhibitorias, pasouse a alimentar o reactor durante todo o período aireado do ciclo. Da estimación das velocidades das distintas etapas da reacción, determinouse que a transferencia de osíxeno desde a fase líquida ao interior do gránulo constituía a etapa limitante. Dado que as BOA teñen maior afinidade polo osíxeno que as BON, estas últimas víronse máis afectadas pola limitación de osíxeno, acumulándose nitrito no sistema. Unha vez descuberto o potencial dos gránulos nitrificantes para producir nitrito, fixouse como obxectivo a obtención da nitrificación parcial, o que implica a oxidación do 50% do amonio a nitrito evitando a oxidación a nitrato. A nitrificación parcial conseguiuse diminuíndo a concentración de osíxeno disolto no medio líquido. Nestas condicións, a máxima carga de nitróxeno parcialmente oxidada foi de 1 g N L^{-1} d^{-1} cunha concentración de osíxeno disolto no rango 2,0—3,5 mg O_2 L^{-1}. Os gránulos mantiveron o seu diámetro medio, a seu integridade e as súas boas características de sedimentación, cun baixo índice volumétrico e unha alta velocidade de sedimentación (100 m h^{-1}) durante a operación do reactor.

No capítulo 4 estudouse a granulación de BON co fin de corroborar que a acumulación de nitrito debíase á competición por osíxeno e non a que as bacterias oxidantes de nitrito presentan máis dificultades para crecer formando agregados que as oxidantes de amonio. O concepto granulación aerobia asóciase en xeral a bacterias heterótrofas, con todo, observouse no capítulo 4 do mesmo xeito que no 3 que as bacterias autótrofas tamén poden desenvolverse formando agregados ou gránulos sen necesidade de ser sometidas a períodos de saciedade e fame. Dado que as BON resultan indesexables nun reactor CANON, pois compiten coas bacterias Anammox polo nitrito, hase de poñer especial atención para evitar o seu desenvolvemento. O obxectivo deste capítulo foi o estudo da evolución da estrutura dos gránulos e das poboacións bacterianas ao cambiar a fonte de nitróxeno alimentada de amonio a nitrito. Os gránulos, cuxo tamaño era de 0,8 mm, estaban colonizados por *Nitrosomonas* (oxidantes de amonio) e *Nitrospira* (oxidantes de nitrito). As bacterias do xénero *Nitrospira* caracterízanse por ser estrategas da k, é dicir, bacterias que teñen maior afinidade polo substrato pero menor velocidade máxima de crecemento que as estrategas da r (*Nitrobacter* no caso das NOB). Unha vez cambiada a fonte de nitróxeno alimentada de amonio a nitrito e tras 250 días de operación non se observou un deterioro da biomasa granular e a poboación de BON maioritaria neste reactor resultou ser a *Nitrobacter*.

No capítulo 5 estúdase o proceso de granulación de bacterias Anammox no tratamento dun efluente sintético salino simulando efluentes industriais, por exemplo as augas residuais xeradas na industria conserveira de peixe. A presenza de concentracións de sal (NaCl) de ata 10 g L^{-1} non só non producía efectos negativos significativos sobre a velocidade de reacción destes microorganismos senón que fomentaba as interaccións entre agregados favorecendo a granulación. O diámetro medio dos gránulos aumentou un 24% e a concentración de biomasa aumentou un 60% logo de engadir NaCl, nunha primeira etapa engadíronse 5 g NaCl L^{-1} na alimentación e na segunda etapa engadíronse 10 g NaCl L^{-1}, proceso que tivo unha duración total de 54 días. As actividades máximas específicas rexistradas foron de 0,45 g N (g VSS)$^{-1}$ d^{-1}. No capítulo 6, estúdase o desenvolvemento do proceso CANON en dous sistemas diferentes: unha columna de borboteo con aireación continua e outra con aireación pulsada. Ambas unidades puxéronse en marcha como reactores nitrificantes nos que se estableceron as condicións de operación axeitadas para levar a cabo a oxidación parcial de amonio nun 50% a unha temperatura de ao redor de 20 ºC.

No reactor con aireación continua a biomasa Anammox desenvolveuse no interior dos gránulos nitrificantes nos que se levaba a cabo o proceso de nitrificación parcial. Malia as condicións adversas presentes no medio líquido: alta concentración de osíxeno disolto e de nitrito, a elevada actividade oxidante de amonio nas capas externas dos gránulos elimina por completo o osíxeno no interior dos mesmos creando

unha zona anóxica rica en amonio e nitrito, propicia para o desenvolvemento de biomasa Anammox (proceso CANON). Nesta unidade a máxima carga tratada foi de 1,1 g N L^{-1} d^{-1} cando o tamaño dos gránulos era de 3,2 mm.

No reactor con aireación pulsada estudouse o arranque dun reactor CANON minimizando o consumo de aire para o aporte do osíxeno requirido. O osíxeno a este reactor aportouse mediante aire pulsado cunha frecuencia de 0.09 s^{-1}. Unha vez desenvolvidos os gránulos nitrificantes cun diámetro de 1,6 mm que levaban a cabo a nitrificación parcial inoculouse o reactor con biomasa pouco enriquecida en bacterias Anammox. Nesta unidade a biomasa Anammox mantívose en forma de agregados mentres que a poboación de BOA permaneceu fundamentalmente en forma de flóculos aínda que unha pequena parte atopábase nas capas máis externas dos gránulos Anammox. O arranque do proceso CANON foi moi rápido e en 35 días a carga de nitróxeno eliminada foi de 0,25 g N L^{-1} d^{-1}, sendo a máxima carga tratada de 0,45 g N L^{-1} d^{-1} cunha concentración de osíxeno de 0,3—0,5 mg O_2 L^{-1}.

No capítulo 7, levouse a cabo un estudo microscópico dos gránulos do reactor CANON con aireación continua utilizando microelectrodos para medir a concentración de osíxeno disolto e de nitrito (os substratos limitantes no proceso CANON). Esta ferramenta combinouse coa técnica FISH de identificación de poboacións bacterianas. Os resultados indicaron unha clara estratificación de poboacións na profundidade dos gránulos con BOA concentradas nunha capa externa de 400 μm, mentres que as bacterias Anammox estaban situadas entre as 400 e as 1000 μm de profundidade. Os resultados da determinación dos perfís de concentración de osíxeno disolto nos gránulos, en experimentos levados a cabo a diferentes concentracións deste no medio líquido, indicaron que o fluxo de osíxeno cara ao interior dos gránulos aumentaba ao aumentar a concentración de osíxeno disolto no medio. Isto demostra que a actividade oxidante de amonio está limitada pola concentración de osíxeno disolto malia que o reactor operouse en condicións próximas ao 100% de saturación con aire. Do mesmo xeito, os perfís de concentración de nitrito realizados variando a concentración deste no medio líquido corroboraron que unha concentración próxima aos 9 mg N L^{-1} é necesaria para que a biomasa Anammox non estea limitada por nitrito. Coa información obtida neste traballo deduciuse que existen uns valores óptimos de concentración de amonio e concentración de nitrito no medio líquido que permiten optimizar a estratexia de control e a operación do proceso CANON.

No capítulo 8, desenvolveuse un modelo capaz de simular para un reactor granular aerobio tanto as concentracións de nutrientes no medio líquido como as propiedades físicas dos gránulos formados, é dicir, os sólidos en suspensión volátiles e a densidade dos mesmos. Para conseguilo, utilizouse un modelo nunha dimensión empregando o software Aquasim e baseado no ASM3 modificado porque se describiu a nitrificación como un proceso en dúas etapas. Con respecto aos demais modelos xa publicados neste tema, incluíronse modificacións necesarias para simular adecuadamente as concentracións e densidade de biomasa que foron a introdución dun perfil de porosidade variable na profundidade do gránulo e a asignación de valores de densidade distintos para bacterias heterótrofas (350 g DQO $(L_{gránulo})^{-1}$) e autótrofas (150 g DQO $(L_{gránulo})^{-1}$). Definíronse no modelo cinco tipos de sustancias sólidas: bacterias heterótrofas, BOA e BON, polímeros de almacenamento e materia inerte e seis tipos de substratos disoltos no medio liquido: osíxeno, DQO, alcalinidade, amonio, nitrito e nitrato.

Cos traballos realizados nesta tese conseguiuse información valiosa para a posible aplicación do proceso autótrofo CANON para eliminar o nitróxeno de efluentes a temperatura ambiente.

Objectives and summary

This thesis is framed in the field of wastewater treatment and more specifically in the field of nitrogen removal from wastewater. The nutrients removal (mainly N and P) from wastewater is necessary in order to avoid the overgrown of algae and the eutrophication of the receiver waters. Nutrients removal is commonly performed by means of biological processes due to the lower cost in comparison with the physicochemical ones. The typical process of nitrogen removal consists on the oxidation of ammonium to nitrate in two steps: 1) the oxidation of ammonium to nitrite by means of ammonia oxidizing bacteria (AOB) and 2) the oxidation of nitrite to nitrate by means of nitrite oxidizing bacteria (NOB). Once the ammonium has been oxidized to nitrite and nitrate, it is reduced to nitrogen gas by the heterotrophic bacteria in absence of oxygen. However, this process is not the most appropriated to treat high nitrogen loaded wastewaters, i.e., wastewaters characterized by low COD/N ratios. In this case, an external carbon source has to be added which increases the cost of the process. An alternative to remove nitrogen from these effluents, which appeared in the last years, is the autotrophic removal of nitrogen carried out by Anammox bacteria which combine ammonium and nitrite to produce nitrogen gas and a small amount of nitrate in anaerobic conditions. This process revealed to be interesting for the treatment of reject water from anaerobic digesters with high nitrogen concentrations and low biodegradable organic matter content. Nevertheless, the slow growth of the AOB in comparison with the heterotrophic ones makes the start-up and the later development of the process difficult. Two alternative configurations are possible to carry out the autotrophic nitrogen removal process: 1) process in two stages: partial nitrification of 50% ammonium to nitrite in the first reactor which fed the second Anammox reactor 2) both processes carried out in a single reactor, process known as CANON ("Complete Autotrophic Nitrogen Removal Over Nitrite").

The autotrophic nitrogen removal from the reject water of anaerobic digesters in wastewater treatment plants presents several advantages such as:

- The increase of nitrogen removal capacity in the plant.
- The reduction of the amount of sludge produced since the productivity of the autotrophic biomass (nitrifying and Anammox) is smaller than the heterotrophic one (denitrifying).
- The production of smaller amounts of greenhouse gases (N_2O and NO).
- The increase of the energy production in the anaerobic digester since, once the nitrogen is removed from the reject water the hydraulic retention time in the primary settlers of the WWTP can be increased. This reduces the aeration energy at similar overall nitrogen removal.

The biological processes can be carried out in systems with suspended biomass or in the form of biofilms (adhered to a carrier material or as granular biomass). The use of systems based on biofilms allows the treatment of larger loads in smaller reactor volumes and therefore, it is recommended for the treatment of effluents with high loads. Furthermore the fact that the sludge is grown as biofilms allows the retention of larger amounts of biomass which is beneficial in the case of slow growing microorganisms such as nitrifying and Anammox bacteria.

During the 90s in The Netherlands, a technology based in aerobic granular biomass was developed. This technology is based on the development of biofilms without the need for support material. The first works

were performed to study the autoaggregation of heterotrophic bacteria fed with a carbon source and the formation of aggregates whose main characteristic is their large settling velocity. To achieve the biomass granulation, a discontinuously operated reactor with a high height/diameter ratio is used (SBR: Sequencing Batch Reactor) and the biomass is exposed to cyclical periods of feast and famine. The use of granular systems presents the following advantages:

- It allows the obtaining of larger biomass concentrations diminishing the sludge yield and increasing the removal capacity of the reactor.
- The biomass washout is minimized, this aspect is important when low growing bacteria are used.
- The biomass settling velocity is much higher than that of activated sludge, with a consequent decrease of the surface needed for the settler (the settler can even be avoided).

On the basis of all the aforementioned, in the present thesis the appropriated operational conditions to obtain the autoaggregation of slow growing organisms like nitrifiers (AOB and NOB) and Anammox in a separated way was studied (Chapters 3, 4 and 5).

Later, the AOB and Anammox populations were combined in a single unit to carry out the CANON process in two different kinds of reactors (Chapter 6). Then with the aim of going deeper in the operation of those granules a microscopic study was performed by means of microelectrodes to follow the concentration profiles inside the aggregates (Chapter 7).

With the experimental results obtained in a previous work, a mathematical model has been developed to simulate the dissolved and particulate compounds of aerobic granules where the nitrogen removal was carried out by nitrification-denitrification (Chapter 8).

The main content of each chapter of the present thesis will be detailed in the following sections.

In Chapter 1 an actualized literature review about the performed studies up to date in the field of nitrogen removal from wastewater is presented. Special attention is paid to autotrophic nitrogen removal (Anammox process). Information regarding aerobic granulation systems, the microbiology of the involved processes and the state of the art of the modelling of this kind of systems is presented.

In Chapter 2, the material and methods used during the different experiments performed along the following chapters are described.

In Chapter 3, nitrifying granules were operated at room temperature (18—24 ºC). These granules were developed in a previous work as heterotrophic granules using acetate as carbon source and then, the organic carbon content in the feeding media was progressively removed until the obtaining of nitrifying granules. With the increase of the nitrogen load applied to the reactor, nitrite accumulated during the operational cycle. This indicated that the rates corresponding to the two nitrification steps (ammonia and nitrite oxidation) were uncoupled at the beginning of the cycle, i.e., when the ammonia concentration had the maximum value. The maximal nitrogen load oxidized to nitrate was of 0.4 g N L^{-1} d^{-1}. When the nitrogen load applied was doubled, a permanent accumulation of nitrite was observed indicating the limitation of the growth rate of NOB in spite of working at dissolved oxygen concentration close to air saturation. With the aim of avoiding high ammonium concentrations in the bulk liquid that could be inhibitory, the reactor was fed during the whole aerated period. Estimations of the rates of the different reaction stages revealed that the oxygen mass transfer from the bulk liquid to the granules was limiting. Since AOB have higher affinity for oxygen than NOB, the last ones were

more affected by oxygen limitation which caused nitrite accumulation in the system. Once the potential of the nitrifying granules to produce nitrite was established, the obtaining of partial nitrification was the objective. Partial nitrification consists of the oxidation of 50% of the ammonium to nitrite avoiding its further oxidation to nitrate. This was achieved by means of decreasing the dissolved oxygen concentration in the bulk liquid. Under these conditions, the maximal nitrogen load partially oxidized was of 0.8 g N L^{-1} d^{-1} with a dissolved oxygen concentration ranging from 2.0 to 3.5 mg O_2 L^{-1}. As a result, the granules kept their mean diameter (around 3 mm), physical integrity and good settling properties, with a low sludge volumetric index and high settling velocity (100 m h^{-1}).

In Chapter 4, the granulation of NOB is studied to corroborate that the nitrite accumulation was caused by a competition for oxygen and that NOB do not present more difficulties to grow in the form of aggregates than AOB. The concept "aerobic granulation" is generally associated to heterotrophic bacteria; however in Chapter 4 as well as in Chapter 3 autotrophic bacteria grew forming aggregates or granules without being exposed to feast and famine periods. Taking into account that NOB are not desired in CANON reactors since they compete for nitrite with Anammox bacteria, special effort has to be made to avoid their growth. The aim of this chapter is the study of the evolution of the granule structure and the bacterial populations after the change of the nitrogen source fed from ammonium to nitrite. Granules with a mean diameter of 0.8 mm were initially colonized by *Nitrosomonas* (AOB) and *Nitrospira* (NOB). Bacteria from the genus *Nitrospira* are "k-strategists", i.e., bacteria with higher substrate affinity but slower maximum growth rate than "r-strategists" bacteria (*Nitrobacter* in the case of NOB). Once the nitrogen source was changed from ammonium to nitrite and after 250 days of operation no damage of the granular biomass was observed and the main population of NOB resulted to be *Nitrobacter*.

In Chapter 5, the granulation process of Anammox bacteria is studied for the treatment of a saline synthetic medium simulating industrial effluent such as wastewater produced in the fish canning industry. The presence of salt concentration (NaCl) up to 10 g L^{-1} did not produce a negative effect on the reaction rate of these microorganisms but it promoted the interactions between aggregates enhancing the granulation. The mean diameter of the granules and the biomass concentration in the reactor increased 24% and 60% respectively after the addition in the feeding of 5 g NaCl L^{-1} and 10 g NaCl L^{-1} in two steps along a period of 54 days. The maximal specific activities registered were of 0.45 g N (g VSS)$^{-1}$ d^{-1}.

In Chapter 6 the development of the CANON process in two different bubble columns is studied: one with continuous aeration and another one with pulsing aeration. Both units were started up as nitrifying reactors where the suitable operational conditions to perform the partial oxidation of 50% of the ammonium to nitrite at around 20 ºC were established.

In the reactor with continuous aeration, the Anammox biomass was developed inside the nitrifying granules where the partial nitrification was also taking place. Despite the adverse conditions present in the liquid media: high dissolved oxygen and nitrite concentrations; the high ammonium oxidation activity in the external layers of the granules fully removed the oxygen in their inner core creating an anoxic zone rich in ammonium and nitrite and suitable for the development of Anammox biomass (CANON process). In this unit the maximal nitrogen removal rate was of 1.1 g N L^{-1} d^{-1} with granules whose mean diameter was of 3.2 mm.

The start-up of a CANON reactor was also studied in a bubble column with pulsing aeration in order to minimize the air consumption in relation to the required oxygen supply. The oxygen was supplied to this reactor by means of air pulsations with a frequency of 0.09 s^{-1}. Once the nitrifying granules were developed with a mean diameter of 1.6 mm and the partial nitrification was established, the reactor was inoculated with biomass

poorly enriched in Anammox bacteria. In this unit, the Anammox biomass was kept in the form of aggregates, while the population of AOB was present mainly in the form of flocs and also in external layers of the Anammox granules. The start-up of the CANON process was fast and in 35 days the nitrogen removal rate was of 0.25 g $N\ L^{-1}\ d^{-1}$. The maximum nitrogen removal rate was of 0.45 g $N\ L^{-1}\ d^{-1}$ at an oxygen concentration of 0.3—0.5 mg $O_2\ L^{-1}$.

In Chapter 7, a microscopic study of the granules inside the CANON reactor with continuous aeration was performed using microelectrodes to measure dissolved oxygen and nitrite concentrations profiles (the two limiting substrates of the CANON process). This tool was combined with the FISH technique which was used to identify the bacterial populations. Obtained results indicated a clear stratification of bacterial populations inside the granules with the AOB concentrated in an external layer of 400 μm, while the Anammox bacteria were placed between 400 and 1000 μm of depth. Experiments performed at different dissolved oxygen concentrations in the bulk liquid revealed that the oxygen flux to the granules increased with the increase of dissolved oxygen concentration in the medium. This result demonstrates that the AOB activity is limited by the dissolved oxygen concentration despite the reactor was operated at conditions close to 100% air saturation. In the same way, the nitrite concentration profiles performed changing the concentration of nitrite in the bulk liquid corroborate that concentrations around 9 mg $N\ L^{-1}$ are needed to avoid the limitation of Anammox bacteria by nitrite. With the information obtained from the present work it is inferred that there exist optimal values of ammonia and nitrite concentrations which allow the optimization of the control strategy and operation of the CANON process.

In Chapter 8, a model able to simulate not only the nutrients concentrations in the bulk liquid but also the physical properties of the formed granules, in terms of volatile suspended solids and density, was developed. The software Aquasim was used to implement a one dimension model developed on the basis of the ASM3 model but modified to describe the nitrification as a two stages process. In comparison to other published models in this field, several modifications were implemented to accurately simulate the concentration and density of the biomass. These modifications consisted of the introduction of a porosity profile in depth inside the granule and the assignation of density values different for heterotrophic (350 g DQO $(L_{granule})^{-1}$) and autotrophic bacteria (150 g DQO $(L_{granule})^{-1}$). In the model five types of solid substances: heterotrophic bacteria, AOB and NOB, storage polymers and inert matter; and six types of substrates in the bulk media: oxygen, chemical oxygen demand, alkalinity, ammonium, nitrite and nitrate were defined

With the work performed in this thesis, important information for the possible application of the autotrophic CANON process for nitrogen removal from wastewater at room temperature was obtained.

Chapter 1

Introduction

Summary

In this chapter, the scope and the motivation of this thesis are detailed and the existent options applied to wastewater treatment are analyzed. This work has been developed in the field of water pollution, specially focused on nitrogen contamination and removal. It is known that the nitrogen cycle is highly affected by anthropogenic activities due to its massive fixation from the atmosphere to produce fertilizers. The release of ammonium in natural systems provokes eutrophication, i.e., proliferation of algae and oxygen depletion in natural waters; this is the reason why nitrogen has to be removed in wastewater treatment plants (WWTP). The traditional way to remove nitrogen from wastewaters is the conventional nitrification-denitrification process. More and more stringent effluent quality requirements and the increasing complexity of wastewaters seriously challenged the efficiency of the classical wastewater treatments.

Recently new technologies have arisen to improve the nitrogen removal from wastewater: bio-augmentation, partial nitrification/denitrification and partial nitrification/Anammox. These processes can be applied to treat reject water (the effluent of the anaerobic digester of the WWTP). The nitrogen removal of the reject water is highly beneficial for the overall nitrogen removal efficiency of the WWTP instead of its direct recirculation to the head of the plant. These new processes will be suitable to implement in new WWTP but they can also be used to improve already built WWTP.

The treatment of reject water by the autotrophic nitrogen removal (which combines partial nitrification and Anammox processes) is highly recommended due to the characteristics of these wastewaters: low concentration of biodegradable carbon source and high concentration of ammonium. The main drawback of the microorganisms involved in the process is their slow growth rate in comparison with heterotrophic bacteria. The application of the granular concept will be analyzed to achieve high nitrogen removal rates due to the high sludge retention times achievable in such systems.

1.1. Water scarcity and water pollution in the world

Despite more than two third parts of earth is made up of water, less than 1% of the water in the planet is available for human consumption and only circa 0.05% is expected to bear water quality standards suitable for drinking. Although water is a renewable resource, the available resources are becoming rapidly polluted. According to the United Nations, 1,100 million people do not have access to safe water while 2,400 million people still lack access to improved sanitation (World Water Assessment Programme, 2009). Compared to the situation in some parts of the world, the status of European water resources is relatively favourable; however, 20% of all surface water in the European Union is seriously threatened with pollution.

Water affects all aspects of human life, from health and sanitation to the food quality; from the environment and ecosystems to the industry and energy that drives the development. The amount of water existent has remained constant for thousands of years while the number and types of users have increased massively. Population growth, urbanization, land use changes, and global warming are creating competing pressures on this finite resource. As a result, the amount of water available for each person is increasingly unequal and diminishing dramatically. It is clear, that urgent action is needed to avoid a global water crisis (World Water Assessment Programme, 2009). Water scarcity and water pollution must be considered as major concerns in order to achieve a sustainable development.

Demography is a specially contributing factor to water scarcity. Over the past 50 years, as the world population rose from 3 billion to 6.5 billion, water use roughly trebled. On current estimates, the population is likely to rise by a further 2 billion by 2025 and by 3 billion by 2050. Demand for water will raise accordingly, or rather, by more, possibly a lot more. It is not the absolute number of people that makes the biggest difference to water use but changing habits and diet. Farmers use about three-quarters of the world water; industry uses less than a fifth and domestic or municipal use accounts for a mere tenth.

Different foods require radically different amounts of water. To grow a kilogram of wheat requires around 1,000 litres. But it takes as much as 15,000 litres of water to produce a kilo of beef. The meaty diet of Americans and Europeans requires around 5,000 litres of water a day to produce. The vegetarian diets of Africa and Asia use about 2,000 litres a day (by comparison, Westerners use just 100-250 litres a day in drinking and washing). Due to this increase in water demand it is important to spare water from the natural resources and to treat the wastewater produced by the anthropogenic action in order to introduce water again in its cycle without compromising its sustainability. Nowadays the removal of pollutants from wastewater, in special organic matter and nutrients like nitrogen and phosphorous is performed in wastewater treatment plants (WWTP) and it is being the object of extensive research.

1.2. Legislation framework

Due to the raising concern about water pollution, new laws are in development to control the quality of the dumped effluents. Since eutrophication process became a serious global problem, there is a need to prevent or substantially reduce negative effects of nutrients on the environment and to involve effective control of nutrient discharges.

Parallel to remarkable advances made in the civilization, particularly brought up by the heavy industrialization period until the end of the 1960s, the major concerns of wastewater treatment were extended to prevent and control degradation of drinking water quality which started to become heavily polluted. For instance, the Environmental Protection Agency (EPA) in the United States was established in 1970 in response

to the growing concerns for degradation of environmental resources: water, air and soil. This was an inevitable result since industrial development at that time completely ignored the impact of the discharge of polluted water to receiving bodies.

In the European Union (EU), the Council Directive 91/271/EEC concerning urban wastewater treatment was adopted to protect the water environment from the adverse effects of discharges of urban wastewater and from certain industrial discharges. There is a general need for secondary treatment of urban wastewater to prevent the environment from being adversely affected by the disposal of insufficiently treated urban wastewater. Member states shall ensure that urban wastewater entering collecting systems should be subjected to secondary treatment or equivalent treatment, before discharge. Moreover, application of more stringent treatment technologies is required in sensitive areas. On 1998, the Commission issued Directive 98/15/EC amending Directive 91/271/EEC to clarify the requirements of the Directive in relation to discharges from urban WWTP to sensitive areas which are subject to eutrophication (Table 1.1). National and European legislations have been introduced to protect environment from human damaging activities. They regulate the maximum allowed concentrations of carbon, nitrogen and phosphorus in purified wastewater discharged to the rivers and other water bodies. A recent directive from EU is the Water Framework Directive (WFD) (2000/60/EC) that regulates the water management throughout the EU.

Table 1.1. Requirements for discharges from urban WWTP to sensitive areas which are subject to eutrophication. One or both parameters may be applied depending on the local situation.

Parameters	Concentration[a]	Minimum percentage of reduction
BOD_5 (mg O_2 L^{-1})	25	70-90 %
COD (mg O_2 L^{-1})	125	75 %
Total suspended solids (mg TSS L^{-1})	35	90 %
Total Nitrogen (mg N L^{-1})	15 (10,000 - 100,000 I.E.) 10 (> 100,000 I.E.)	70-80 %
Total Phosphorous (mg P L^{-1})	2 (10,000 - 100,000 I.E.) 1 (> 100,000 I.E.)	80 %

[a]Information extracted from Council directive (91/271/EEC) amended by the Commission directive 98/15/EC. (I.E.: inhabitant equivalent)

In Galicia, an autonomous community in northwest Spain, in the last years, the local legislation tried to implement the European directives corresponding to wastewater treatment and effluent quality and specially the directive 91/271. On 2000, a sanitation program for the period 2000 - 2015 (Xunta de Galicia, 2000) was established and sensitive areas of the community were defined (Fig. 1.1). Only one zone was defined as "sensitive area"; the Ria of Pontevedra. The parts of the coast where the seawater has sufficient velocity to avoid the accumulation of nutrients are defined as less sensitive areas and the rest (rivers, lakes, rias, etc.) were defined with a normal level of sensitiveness.

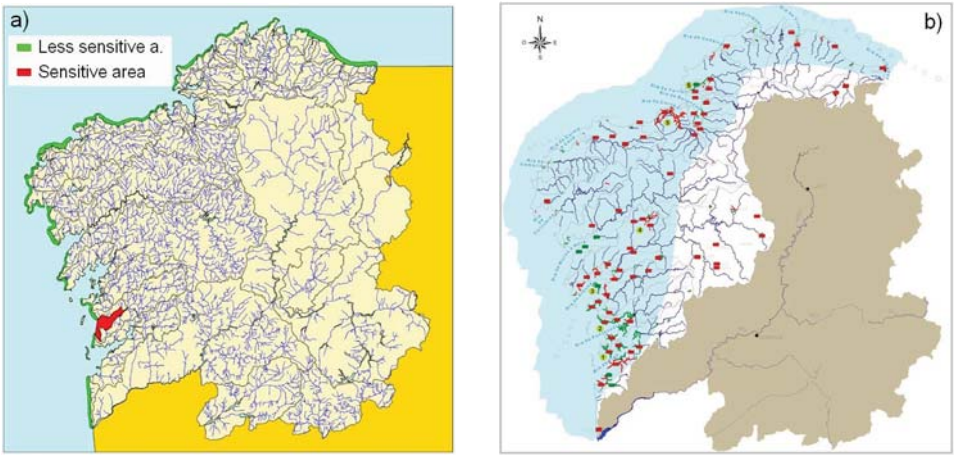

Figure 1.1. a) Definition of sensitive (red) and less sensitive (green) areas in Galicia. b) WWTP and piping in project (green) and under construction in 2000 (red) in the coast of Galicia.

The contamination caused in Galicia by wastewater pollution was estimated and evaluated in terms of inhabitant equivalent as indicated in Table 1.2.

Table 1.2. Distribution of the wastewater pollution in Galicia.

	Inhabitant Equivalent	% Pollution source
Population of Galicia	2 720 445[a]	47 %
Estimated seasonal population	444 916	7 %
Estimated industrial pollution	2 025 114	46 %
Total	5 990 475	100 %

[a]Data from 1991

There was still in year 2000 a lot to do in Galicia since the wastewater treated corresponded to 2.5 millions I.E. but some of these treatments were not biological. In January 1999 the 43 % of the WWTP were not accomplishing the required quality of the effluent. It can be observed in Fig. 1.2 that only two thirds of the operating WWTP were equipped with a biological treatment. At this point the constructed WWTP of the big cities were having troubles since the WWTP of Lugo, Vigo, and Ourense were overloaded and the WWTP of A Coruña (550,000 I.E.) and Pontevedra (107,000 I.E.; this one dumping at the Ria of Pontevedra defined as sensitive area) were only equipped with primary treatment.

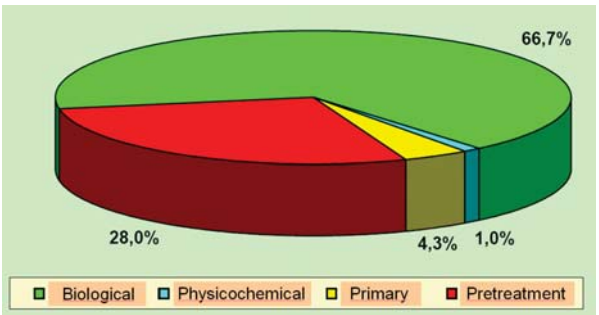

Figure 1.2. Treatments installed in the WWTPs of Galicia in 2000.

In order to fulfill the directives and the Water Framework Directive set up by the EU, different WWTP were under construction in 2000 whereas many others were projected (Fig 1.1b); all these construction should be finished in 2015.

Nutrient presence in wastewater represents and important pollution source which has to be removed from the generated effluents to reduce the detrimental effects on the environment. In most cases the nutrients are removed from wastewaters by means of biological treatments.

1.3. Presence of nitrogen compounds in the environment

The turnover of nitrogen compounds in the biosphere is known as the nitrogen cycle (Fig 1.3). Most biologists assumed years ago that the microbial nitrogen cycle was essentially complete (Strous and Jetten, 2004). However, the last years indicated that its understanding is far from complete. Discoveries such as, e.g., anaerobic ammonium oxidation (Anammox) (Strous et al., 1999b), ammonium oxidation by crenarchaea (Konneke et al., 2005), or genome sequencing of several N-cycle organisms (Strous et al., 2006) provide examples that there is an enormous biodiversity of nitrogen conversions hidden in the microbial world. With the discovery of Anammox bacteria, besides new alternatives to nitrogen removal in WWTP, the thought that the denitrification in the oceans was mainly due to heterotrophic bacteria changed substantially. Anammox bacteria revealed to be prevalent in low-O_2 marine environments as the Black Sea (Kuypers et al., 2003) or the Benguela upwelling system (Kuypers et al., 2005). The contribution of the process to N_2 production is however strongly site specific (Revsbech et al., 2006) and range between a few percent in some shallow water sediments (e.g. Trimmer et al., 2003) to almost 100% in the water column at the Benguella upwelling region (Kuypers et al. 2005).

Current estimates suggest that globally Anammox may be responsible for 30–50% of N_2 production in the ocean but with further research in different oceanic zones with minimum oxygen presence this percentage could be much higher.

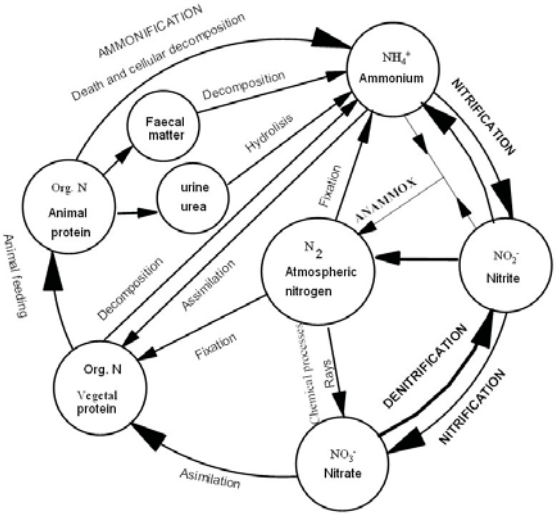

Figure 1.3. Nitrogen cycle.

There is probably no other elemental cycle, where the human impact has been as dramatic as it is the case for nitrogen. Recent reports indicate that mankind continues to transform the global nitrogen cycle at a record pace, by increased combustion of fossil fuels and increased demand for nitrogen in agriculture and industry (Galloway et al., 2008). Much of this anthropogenic nitrogen is lost to the environment and causes a cascade of problems, most notably an increase in freshwater nitrate levels and increased nitrous oxide production that may enhance global climate change (Duce et al., 2008).

The biological systems in nature have "learned" to operate in a resource and energy efficient way via the driving force of evolutionary competition and selection. Once nitrogen has entered the biosphere via biological fixation, it is subjected to a series of conversion steps, from plant protein to animal protein, and finally ends up in dead organic matter. When this organic matter is mineralised in the soil, most of the inorganic nitrogen compounds produced (ammonium, nitrite, nitrate) are taken up again by plant or microbial biomass for the production of protein. From the nitrogen fluxes in Figure 1.4a, we can calculate that the average retention time of fixed nitrogen in the biosphere is over 7,000 years before it is eventually recirculated to atmospheric nitrogen.

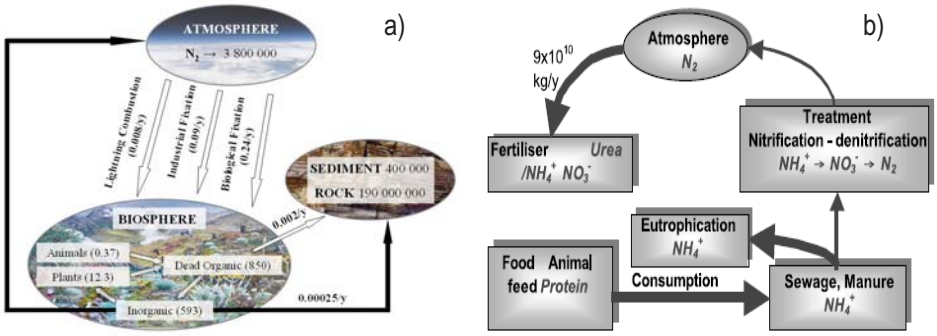

Figure 1.4. Main fluxes and compartments in a) the natural nitrogen cycle (values x 10^{12} kg) and b) the anthropogenic nitrogen cycle (adapted from Gijzen, 2001).

However, the world annual industrial production of nitrogenous fertiliser has increased sharply. During the period 1960 to 2004 the world population doubled, while the production of fertiliser increased almost ten-fold, from 0.01 to 0.09 x 10^{12} kg. Current production constitutes about 37% of the total amount of nitrogen input achieved via terrestrial and marine biological N_2 fixation (about 0.24 x 10^{12} kg per year).

Intensive agriculture requires the use of fertilisers, which are obtained via industrial fixation of atmospheric nitrogen via the Haber-Bosch process. This process combines nitrogen and hydrogen gases (Eq. 1.1) using a form of magnetite, iron oxide, as the catalyst (at 150–250 bar and 300-550 °C).

$$N_2\,(g) + 3\,H_2\,(g) \rightleftharpoons 2\,NH_3\,(g) \qquad (1.1)$$

Only a fraction of the total amount of inorganic nitrogen is recirculated to N_2 via nitrification/denitrification. As a result, the total influx of nitrogen into the biosphere is much larger than the nitrogen removal rate via denitrification (Fig. 1.4b). It may not be surprising therefore that we see widespread eutrophication of fresh water resources worldwide.

The recovery of ammonia in WWTP instead of its removal was highlighted as an interesting alternative to decrease the industrial fixation of N. However, its recovery in WWTP is not interesting from an economical point of view since it is much cheaper to fix it from the atmosphere than to purify it from wastewater. This is not the case with phosphorous, since easily accessible global mineral phosphorus reserves are limited which is not the case with atmospheric nitrogen.

The presence of anthropogenic generated nitrogen compounds in the environment creates important problems of toxicity or eutrophication of natural water sources.

1.3.1. Toxicity of nitrogen compounds

Large concentrations of ammonium, nitrite and nitrate in the nature are toxic for life. Free ammonia can have toxic effects on aquatic life; these effects may be either acute (i.e. fish mortality) or chronic (impacts on reproduction, tumours, etc.). Concentrations of ammonia as low as 0.03 mg NH_3-N L^{-1} were found to be toxic to aquatic organisms (Solbe and Shurben, 1989).

Nitrite is manufactured mainly for use as a food preservative, and both nitrate and nitrite are used extensively to enhance the colour and extend the shelf-life of processed meats. Nitrite can bind to iron on hemoglobin reducing transfer of oxygen to cell tissues, the result is suffocation accompanied by bluish tinge of the skin. Nitrite and nitrate in drinking water have been medically linked to methemoglobinemia, a sometimes fatal blood disorder affecting infants ("blue baby syndrome"). Nitrate can be reduced to nitrite under certain conditions in the stomach and saliva.

Nitrate is primarily used to make fertilizer, although it is also used to make glass and explosives and other chemical production and separation processes. Excess of nitrate in the soil is most often found in rural and agricultural areas. Nitrate travels easily through the soil, carried by rain or irrigation water into groundwater basins. Nitrate pollution impedes the production of drinking water. During chlorination of drinking water; carcinogenic nitrosamines may be formed by the interaction of nitrite with compounds containing organic nitrogen.

1.3.2. Eutrophication

Overloading with nutrients of natural water sources can cause health problems for fishes, animals and humans and can also stimulate growth of algae that later on lead to disturbances in the water system. The substrates required for algae growth are: nitrogen, phosphorus, carbon dioxide, light and micronutrients. Phosphorus is typically the limiting factor for algae growth in rivers and lakes whereas nitrogen can be a limiting factor for algae growth in estuaries. The primary cause of eutrophication is an excessive concentration of plant nutrients originated from agriculture or sewage treatment. Algal blooms are influencing the water system in two ways. First, they hamper the penetration of sunlight, causing death of underwater grasses. Secondly, the decomposition of died algae causes depletion of oxygen, which is normally essential to most organisms living in water. Moreover, excess of nutrients and eutrophication creates uncomfortable conditions as odours that influence the recreational use of lakes and estuaries. In addition, aquatic plants (including algae) influence the oxygen and pH of the surrounding water. The greater the growth of algae, the greater the fluctuations in levels of dissolved oxygen and pH. This can upset metabolic processes in organisms, which can result in disease or death.

In some specific cases, local authorities must rely on eutrophic waters to produce drinking water. There are two major risks for health in using such waters:

1. Risks linked to the presence of organic matter: the treatment of raw water with high levels of organic matter is always technically difficult. It can lead to the production of carcinogenic by-products (trihalomethanes, other chlorinated components or ozonides) as a result of their reaction with disinfectants.

2. Risks linked to the presence of specific cyanobacteria in fresh waters: when eutrophication leads to the development of cyanobacteria that are potentially toxic, the elimination of these toxins is complex.

1.4. Nitrogen removal from wastewater

The nitrogen compound mainly present in wastewaters is ammonium (NH_4^+) which can be removed by physicochemical or biological processes. The selection of the best alternative is generally based on cost-effectiveness issues. However, in practice the selection of either a biological or a physiochemical method is determined by the nitrogen concentration of the wastewater. Three concentration ranges can be distinguished (Mulder, 2003):

a) Diluted wastewater with ammonium concentration up to 100 mg N L^{-1} (e.g. domestic wastewater). In this range biological processes such as activated sludge are preferred processes based on cost-effectiveness issues.

b) Concentrated wastewater with ammonium concentrations in the range of 100-5000 mg N L^{-1}. A typical example is sludge reject water for which, after extensive investigations, biological treatment is to be preferred. The main reason to select a biological process is the lower price compared to the physicochemical methods. Van Dongen et al., (2001) estimated a cost of 4.5–11.3 euros per kg N removed with physicochemical techniques and 2.3–4.5 euros per kg N removed using conventional biological processes (nitrification-denitrification) for the case of The Netherlands. Recently new biological nitrogen removal processes for these concentrated streams have been under study as it is the case of the autotrophic nitrogen removal which can be carried out in two steps (e.g., the SHARON-Anammox process, van Dongen et al., 2001), or one single step (e.g., the CANON process, Third et al., 2001).

c) Concentrated wastewater with ammonium concentrations higher than 5000 mg N L^{-1}. In this range physicochemical methods are technically and economically feasible. The main physicochemical processes applied for ammonium removal are air stripping; breakpoint chlorination and selective ion exchange. The last two options have the inconvenient of involving technologies with high operational costs and complex control. Ammonia stripping is widely used because of its simple operation and high efficiency.

When biological processes are considered, besides the ammonium concentration in wastewater, its COD/N ratio will determine the most suitable biological process to carry out nitrogen removal:

1) COD/N > 20: in this case, the assimilation of nitrogen by heterotrophic bacteria is sufficient to remove nitrogen.

2) 20 < COD/N < 5: removal of nitrogen by assimilation and nitrification-denitrification pathway.

3) COD/N < 5: in this case, the nitrification-denitrification process is not suitable since an additional carbon source is needed. Nitrogen removal by “nitrite-route” processes such as partial nitrification-denitrification or partial nitrification-Anammox are being implemented to optimize WWTP operation. The removal of nitrogen from these “problematic” wastewaters will be the aim of the thesis.

1.5. Nitrification-denitrification processes

Biological processes in wastewater treatment are mainly carried out by bacteria. Conventionally, nitrogen removal from WWTP is carried out by the nitrification-denitrification process. It is based on a sequence of aerobic and anoxic conditions where the ammonium is firstly oxidized to nitrate in the presence of oxygen by nitrification and then reduced using an organic carbon source as electron donor during the denitrification process.

1.5.1. Nitrification

Nitrification, which converts ammonia first to nitrite and then to nitrate, is the initial step of biological nitrogen removal processes carried out by two phylogenetically independent groups of autotrophic aerobic bacteria, namely, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). The nitrification process is carried out in two sequential stages: the ammonium oxidation to nitrite and the subsequent oxidation of nitrite to nitrate.

The oxidation of ammonium to nitrite by AOB produces hydroxylamine as intermediate. The enzymes involved are ammonia monooxygenase for ammonium oxidation (Eq. 1.2) and hydroxylamine oxidoreductase to produce nitrite (Eq. 1.3) with the complete process as described in Eq. 1.4.

$$NH_4^+ + O_2 + H^+ + 2\ e^- \rightarrow NH_2OH + H_2O \quad (1.2)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 4\ e^- + 5\ H^+ \quad (1.3)$$

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \quad (1.4)$$

The carbonate system is usually the pH buffer available in the wastewater which neutralizes the production of protons through CO_2 stripping (Eq. 1.5).

$$NH_4^+ + 1.5\ O_2 + 2\ HCO_3^- \rightarrow NO_2^- + 3\ H_2O + 2\ CO_2 \quad (1.5)$$

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

$$NH_4^+ + 1.382\ O_2 + 1.982\ HCO_3^- \rightarrow 0.018\ C_5H_7O_2N + 0.982\ NO_2^- + 2.927\ H_2O + 1.891\ CO_2 \quad (1.6)$$

As represented in the Eq. 1.6, 2 moles of alkalinity are removed due to CO_2 stripping per mole of ammonium oxidized due to the release of protons during the ammonium oxidation (Eq. 1.5). In the cases when this amount of buffer is not available in the water, the pH of the medium drops and the ammonium oxidation rate decreases sharply.

In the second stage of the nitrification process, nitrite is oxidized to nitrate by NOB by means of the enzyme nitrate oxidoreductase (Eq. 1.7).

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (1.7)$$

When including bacterial growth in the stoichiometry the Eq. 1.8 is obtained.

$$NO_2^- + 0.003\ NH_4^+ + 0.488\ O_2 + 0.010\ H_2CO_3 + 0.003\ HCO_3^- \rightarrow 0.003\ C_5H_7O_2N + NO_3^- + 0.008\ H_2O \quad (1.8)$$

Taking into account the two steps of the nitrification process, several aspects have to be pointed out (by comparison of Eq. 1.6 and 1.8): 1) ammonium oxidation to nitrite consumes more oxygen than nitrite oxidation;

for the first step of ammonium oxidation 3.16 g O_2 are required to oxidize 1 g NH_4-N to nitrite while for the second step 1.11 g O_2 are needed to oxidize 1 g NO_2-N to nitrate. 2) ammonium oxidation produces more bacteria per mole of nitrogenous compound oxidized. 3) ammonium oxidation to nitrite produces protons, to oxidize 1 mole (14 mg N) ammonium to nitrate 2 mol of alkalinity are consumed.

1.5.1.1. Parameters affecting nitrification

The main parameters affecting the nitrification process are: the concentrations of dissolved oxygen (DO) in the bulk liquid, the concentrations of substrates: ammonium and nitrite, the temperature and the pH. The growth rate (μ) of the microorganisms can be described by a Monod equation that indicates the dependency of the bacteria growing on a limiting substrate S, including a bacteria decay coefficient (Eq. 1.9).

$$\mu = \mu_{max} \cdot \frac{C_S}{K_S + C_S} - b \qquad (1.9)$$

where: μ = growth rate (d^{-1})

μ_{max} = maximum microorganisms growth rate (d^{-1})

K_S = half saturation constant (g L^{-1})

C_S = limiting substrate concentration (g L^{-1})

b = decay constant (g g^{-1} d^{-1})

The activity of the nitrifying bacteria begins at temperatures around 4 ºC, its maximum growth rate increases with the temperature up to the maximum value at temperatures around 35-40 ºC. At temperatures higher than 40 ºC the activity falls sharply.

The concentrations of ammonium and nitrite can either limit or inhibit the nitrification process and for this reason they have to be controlled. The half saturation constants of ammonium and nitrite for AOB and NOB are 1 mg NH_4^+-N L^{-1} and 1 mg NO_2^--N L^{-1}, respectively (Henze et al., 2000). Therefore ammonium and nitrite concentrations higher than 1 mg N L^{-1} would be necessary in order to avoid substrate limitation of the nitrification process. However, too high concentrations of ammonium and nitrite and specially their unionized forms: free ammonia and free nitrous acid inhibit the process and therefore they must be controlled, further information about AOB and NOB inhibitions will be given in the next section.

The significance of pH decrease during the nitrification process relies on the fact that the reaction rates are rapidly depressed as the pH is reduced below 7.0 (Fig. 1.5). Therefore, in cases where the alkalinity of the wastewater will be depleted by the acid produced by nitrification, the proper amount of alkalinity must be supplemented by a chemical addition (EPA, 1993) to avoid a drop in the nitrifying activity.

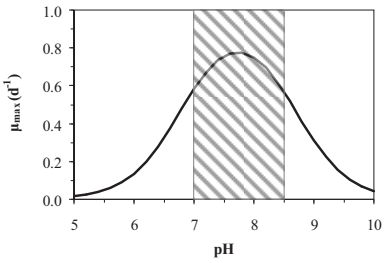

Figure 1.5. Representation of the μ_{max} of AOB and NOB vs pH at 20 ºC (obtained from data published Jubany et al., 2008).

The pH buffer normally available in wastewaters is the inorganic carbon. The equilibrium of the carbonate system in water plays a key role in both processes: nitrification and denitrification and furthermore it depends strongly on the pH of operation. Carbonic acid (H_2CO_3) is a diprotic acid which corresponds to dissolved carbon dioxide; this acid can yield more than one proton in different equilibriums (Eq. 1.10).

$$CO_2\,(g) + H_2O \rightleftharpoons H_2CO_3\,(aq) \rightleftharpoons H^+ + HCO_3^-\,(aq) \rightleftharpoons 2\,H^+ + CO_3^{2-}\,(aq) \qquad (1.10)$$

The ratio of the different acid/base species at different pH is represented (Fig. 1.6). Since bacterial activity occurs around neutral pH, the main carbonate compound in wastewater will be the bicarbonate ion. At pH 7, around 20% of the inorganic carbon in solution corresponds to H_2CO_3 which is in equilibrium with gaseous CO_2. The hydration equilibrium constant of CO_2 and the Henry constant both at 25°C are $1.70 \cdot 10^{-3}$ and 29.76 atm l mol^{-1}, respectively: hence, the majority of the carbon dioxide is not converted into carbonic acid and stays as CO_2 molecules.

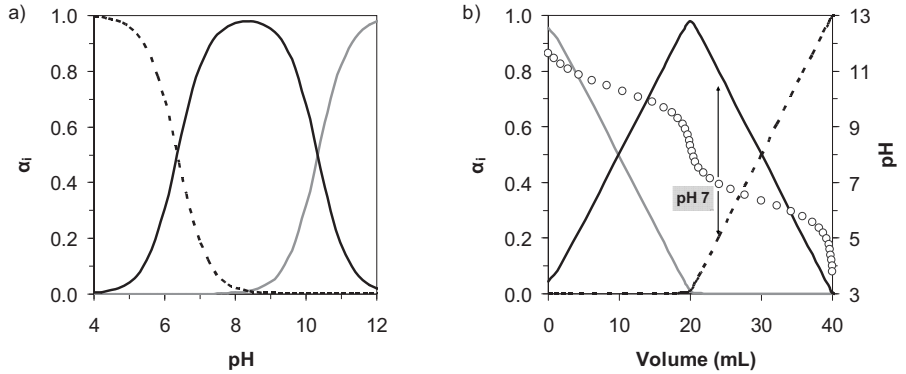

Figure 1.6. a) Molar fractions of H_2CO_3 (**- - -**), HCO_3^- (**—**) and CO_3^{2-} (—) in solution at different pH values (T = 25 ºC). b) Distribution of acid/base species: H_2CO_3 (**- - -**), HCO_3^- (**—**) and CO_3^{2-} (—) and pH (○) along a titration curve of 20 ml of CO_3^{2-} 0.1 M with HCl 0.1 M (performed with the computational freeware program CURTIPOT available in Internet).

1.5.1.2. Microbiology of AOB and NOB

Although the basic metabolism is more or less uniform for all ammonia-oxidizing bacteria, different physiological requirements exist among the different strains (Wagner et al., 1995; Koops and Pommerening-Roser, 2001). For example, the substrate affinity, salt requirement and salt tolerance differ significantly among ammonia oxidizers. AOB are classified in two phylogenetic groups. One group is related to the γ subclass of the Proteobacteria. Its only genus, *Nitrosococcus*, is represented by two described marine species (Koops and Pommerening-Roser, 2001). The second group belongs to the β subclass of the Proteobacteria. Two clusters exist within this assemblage, the *Nitrosospira* cluster (with three genera) and the *Nitrosomonas* cluster. All members of the three genera of the *Nitrosospira* cluster are very closely related to each other, whereas the *Nitrosomonas* cluster reveals at least five distinct lineages of descent (Koops and Pommerening-Roser, 2001). Community studies on wastewater treatment systems have indicated that *Nitrosomonas* strains form one of the most important populations. Three lineages (*Nitrosomonas europaea, Nitrosomonas oligotropha,* and *Nitrosomonas communis* lineages) are routinely observed as dominant organisms in wastewater treatment plants (Gieseke et al., 2001).

Nitrite oxidizing bacteria also differ in their physiological requirements and capabilities; they have been classified into four groups. The major group, which belongs to the α subclass of Proteobacteria, is represented by a single genus, *Nitrobacter*, with four described species (Sorokin et al., 1998). The two species of the genus *Nitrospira*, *Nitrospira marina* and *Nitrospira moscoviensis*, are members of a distinct phylum close to the δ subclass of *Proteobacteria*. In contrast to textbook knowledge, *Nitrospira*-like bacteria and not *Nitrobacter* spp., are the dominant nitrite oxidizers in most full-scale wastewater treatment plants, in laboratory scale reactors and also in different environmental samples (Daims et al., 2000). These findings seem to suggest that *Nitrospira* are widely distributed in nature and probably contribute significantly to global nitrite oxidation.

1.5.2. Partial nitrification

In the last years, new strategies arose focused on the development of a shortcut in the nitrogen cycle named the "nitrite route", which avoids the nitrite oxidation to nitrate. In this case, the further denitrification of the produced nitrite to nitrogen gas could be performed under autotrophic or heterotrophic conditions. To obtain partial nitrification, the ammonium has to be converted into nitrite by the AOB while the oxidation of nitrite to nitrate carried out by NOB has to be avoided. The AOB and NOB are two phylogenetically unrelated groups whose different growth rates and the way their growth rates are affected by parameters like temperature, pH, dissolved oxygen (DO), etc. can be used to outcompete NOB and to uncouple both reaction rates.

The oxidation of high ammonium concentrations causes a significant pH-decrease, which limits further ammonium conversion as aforementioned. Sludge reject water typically contains equimolar amounts of bicarbonate and ammonium, so half of the produced protons are neutralized by CO_2-stripping. As a result, for streams containing bicarbonate and ammonium in equimolar amounts and without additional pH control in the reactor, typically half of the ammonium is converted before a significant pH-decrease occurs, that prevents further ammonium oxidation.

Since the different growth parameters for AOB and NOB are differently affected by the operational conditions, the conditions where AOB grow faster than NOB can be selected ($\mu_A > \mu_N$). According to this, several strategic parameters have been controlled in WWTP to reach partial nitrification:

1) Temperature and SRT: At the usual temperatures of operation of WWTP no nitrite build-up is registered. This is due to higher growth rate of NOB in the range of temperatures from 10 to 20 ºC. However the oxidation of ammonia has higher activation energy than the oxidation of nitrite. Therefore, the operation at temperatures above 25 ºC would allow the washout of NOB which grow slower than AOB (Hellinga et al., 1998) (Fig. 1.7).

2) Dissolved oxygen: AOB have lower oxygen affinity than NOB (Table 1.3). By controlling the DO concentration to low values, the oxidation of nitrite to nitrate can be controlled. Nitrite accumulation was, for instance, obtained manipulating the dissolved oxygen concentration in biofilm systems (Garrido et al., 1997; Bernet et al., 2005).

Cecen and Gonenc, (1995) found that the bulk oxygen to bulk ammonia ratio is the most crucial parameter in the accumulation of nitrite. In nitrification, these researchers found a considerable degree of nitrite accumulation at bulk O_2/bulk NH_4^+ ratios lower than 5 mg O_2 (mg N)$^{-1}$. Bernet et al., (2005) found that using O_2/NH_4^+ ratio set points of 0.05 and 0.1 mg O_2 (mg N)$^{-1}$ it was possible to oxidize up to 80% of the inflow NH_4^+ into NO_2^-.

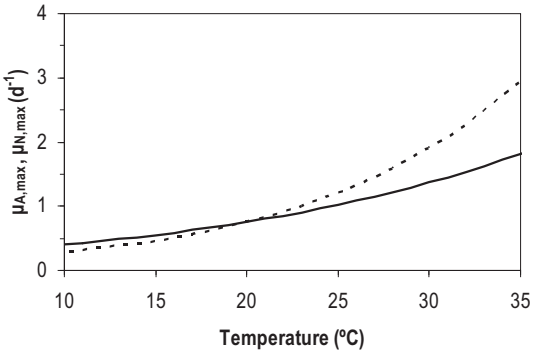

Figure 1.7. Comparison of the evolution of the μ_{max} of AOB (- - -) and NOB (▬) with the temperature for a pH value of 7.5 (obtained from data published by Jubany et al., 2008).

Table 1.3. Comparison of oxygen affinity constants of AOB (K_{O2}^{A}) and NOB (K_{O2}^{N}) at different temperatures.

K_O^A (mg L^{-1})	K_O^N (mg L^{-1})	T (ºC)	Reference
0.60	1.30	20	Wiesmann, (1994)
0.30	1.10	25	Wiesmann, (1994)
0.72	1.75	25	Guisasola et al., (2005)
0.50	1.00	30	Pambrun et al., (2006)
0.24	1.50	30	Wyffels et al., (2004)
0.25	0.50	35	Dold et al., (2007)

3) Free ammonia (FA) and free nitrous acid (FNA) concentrations: Despite the mechanisms of inhibition of FA and FNA remain unclear (Vadivelu et al., 2007), *Nitrobacter* have been described to be more sensitive to FA and FNA inhibitions than *Nitrosomonas*. Anthonisen et al., (1976) proposed the expressions from Eq. 1.11 and 1.12 to calculate the concentrations of NH_3 and HNO_2 at the operational temperature (T) from the NH_4^+, NO_2^- concentrations and the pH value in the bulk liquid.

$$C_{NH_3} = \frac{C_{NH_4}}{\left(\frac{e^{\frac{6344}{T+273}}}{10^{pH}} + 1\right)} \tag{1.11}$$

$$C_{HNO_2} = \frac{C_{NO_2}}{\left(10^{pH} e^{\frac{-2300}{T+273}}\right)} \tag{1.12}$$

Anthonisen et al., (1976) reported that FA initiated inhibition on *Nitrobacter* at about 0.1–1.0 mg NH_3-N L^{-1}, while the threshold value for *Nitrosomonas* was about 10–150 mg NH_3-N L^{-1}. They also reported that FNA at a concentration of as low as 0.22 mg HNO_2-N L^{-1} inhibited the activities of *Nitrobacter*.

More recently, Vadivelu et al., (2006a, 2006b, 2007) uncoupled the effect of both inhibitors in the anabolism and catabolism of *Nitrosomonas* and *Nitrobacter* performing tests in the presence or absence of inorganic carbon. Again, Nitrobacter growth (anabolism) was inhibited at much lower values of FA and FNA than *Nitrosomonas*. The biosynthesis was completely stopped at a FNA concentration of 0.40 mg HNO_2-N L^{-1}

for *Nitrosomonas* and 0.023 mg HNO_2-N L^{-1} for *Nitrobacter*. FA concentrations up to 16.0 mg NH_3-N L^{-1} (the highest concentration tested) did not have any inhibitory effect on either the catabolic or anabolic processes of the *Nitrosomonas* culture while *Nitrobacter* likely ceased to grow at a FA level of above 6 mg NH_3-N L^{-1}. The adaptation of bacteria to inhibitions after long term operation is the main drawback of a strategy based exclusively on this factor (Turk and Mavinic, 1989).

1.5.3. Denitrification

In the denitrification process, the nitrate and/or nitrite present in the wastewaters is reduced to molecular nitrogen in anoxic conditions by the action of heterotrophic bacteria. The process requires the presence of a source of organic carbon as electron donor, e.g. acetic acid or methanol, and nitrate acts as the last electron acceptor in the respiratory chain substituting the O_2 molecule. The reduction is carried out by subsequent steps through different oxidation states of nitrogen (Eq 1.13 to 1.16) and the global stoichiometry with acetic acid as organic carbon source is represented in Eq. 1.17.

$$2\ CH_3COOH + 8\ NO_3^- \rightarrow 4\ CO_2 + 8\ NO_2^- + 4\ H_2O \quad (1.13)$$

$$CH_3COOH + 8\ NO_2^- + 2\ H_2O \rightarrow 2\ CO_2 + 8\ NO + 8\ OH^- \quad (1.14)$$

$$CH_3COOH + 8\ NO \rightarrow 2\ CO_2 + 4\ N_2O + 2\ H_2O \quad (1.15)$$

$$CH_3COOH + 4\ N_2O \rightarrow 2\ CO_2 + 4\ N_2 + 2\ H_2O \quad (1.16)$$

$$5\ CH_3COOH + 8\ NO_3^- \rightarrow 10\ CO_2 + 4\ N_2 + 6\ H_2O + 8\ OH^- \quad (1.17)$$

Rewriting equation 1.17 taking into account the equilibrium of CO_2 gives Eq. 1.18.

$$NO_3^- + 0.625\ CH_3COOH \rightarrow HCO_3^- + 0.25\ CO_2 + 0.5\ N_2 + 0.75\ H_2O \quad (1.18)$$

From the stoichiometry it can be inferred that the denitrification process originates an increase in the medium alkalinity and that 40% of the organic matter needed is used to reduce nitrate to nitrite.

1.5.3.1. Microbiology of Denitrifiying bacteria

This process makes use of N oxides (e.g. nitrate or nitrite) as terminal electron acceptors under anaerobic, microaerophilic, and occasionally aerobic conditions. Important advances in the biochemical characterization of denitrification and the underlying genetics have been achieved with various organisms (like species from the genera *Pseudomonas*, *Paracoccus*, *Ralstonia*, and *Rhodobacter*). Around 50 genes are required within a single bacterium for the synthesis of the biochemical denitrification apparatus (Zumft, 1997).

1.5.4. Anaerobic ammonium oxidation (Anammox)

Another possibility to remove ammonium from wastewaters with low COD/N ratios consists of the autotrophic nitrogen removal combining AOB and Anammox bacteria. For a long time, it was thought that ammonium oxidation could only take place aerobically. Broda (1977) predicted, using thermodynamic calculations, the existence of chemolitoautotrophic bacteria capable to oxidize ammonium using nitrite as electron acceptor. That prediction would be experimentally confirmed two decades later by Mulder et al., (1995) in a denitrifying pilot plant, treating wastewaters from a yeast plant. Anammox bacteria convert ammonium together with nitrite (electron acceptor) directly to dinitrogen gas in the absence of any organic carbon source, following the reaction described in Eq. 1.19 (Strous et al., 1998). In this process a small amount of nitrate is also produced in the anabolism of Anammox bacteria.

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \quad (1.19)$$

Anammox bacteria have been detected in several wastewater treatment plants all around the world; they belong to the genus *Plantomycetes* and their optimal temperature and pH of operation are 35 ºC and 8, respectively. These bacteria are characterised by a low productivity: 0.038 g VSS (g N)$^{-1}$ and a slow growth rate with large doubling times as long as 11 d (Strous et al., 2002). The advantage of this low biomass productivity is the reduction of operation costs related to sludge handling in the WWTP. On the other hand, the slow growth rate of Anammox bacteria makes the start up of the Anammox processes long and difficult. It is therefore mandatory to start-up Anammox processes in reactors with good biomass retention. Another important characteristic of Anammox bacteria is the fact that they are inhibited by both O_2 and NO_2^-. Anammox bacteria are inhibited by an oxygen partial pressure of only 0.5% of air saturation, however, this inhibition is reversible once anaerobic conditions are re-established (Strous et al., 1997). Related to NO_2^- inhibition, no uniformity is found in the literature about the threshold values. For instance, Strous et al., (1999a) reported a value of 100 mg N L^{-1} as completely inhibitory whereas Dapena-Mora et al., (2007) reported that concentrations of nitrite of 350 mg N L^{-1} corresponded only to 50% inhibition of Anammox bacteria. Further studies are needed in order to clarify this point. What is known is that, it is mandatory to maintain nitrite to low values in order to avoid the total breakdown of the process since once nitrite begins to accumulate there is high risk of failure in the reactor (Dapena-Mora et al., 2004a).

1.5.4.1. Microbiology of Anammox bacteria

Since the discovery of Anammox process in Delft (The Netherlands), evidence for Anammox activity has been obtained, in a variety of laboratory and engineered systems (Schmid et al., 2005) besides the natural environments aforementioned. Until now, several Anammox organisms were identified: *Candidatus* "Brocadia Anammoxidans", *Candidatus* "Kuenenia stuttgartiensis", *Candidatus* "Scalindua sorokinii", *Candidatus* "Scalindua brodae", *Candidatus* "Scalindua wagneri", *Candidatus* "Brocadia fulgida" and *Candidatus* "Anammoxoglobus propionicus" and their 16S rRNA sequences were determined (Schmid et al., 2000; Fujii et al., 2002; Schmid et al., 2003; Kartal et al., 2007; Kartal et al., 2008).

The studies about enrichment or operation of the Anammox process at laboratory or pilot scale reactors up to date were performed mainly with bacteria of the genus *C.* "Brocadia" or *C.* "Kuenenia", so the major part of the available information on Anammox is about these types of bacteria. The main difference between *Candidatus* "K. stuttgartiensis" and *Candidatus* "B. anammoxidans" is their Anammox activity: 26.5 nmol N_2 (mg protein)$^{-1}$ min^{-1} at pH 8 and 37 ºC for C. "Kuenenia" (Egli et al., 2001) and 55 nmol N_2 (mg protein)$^{-1}$ min^{-1} at pH 8 and 40 ºC for "Brocadia" . However, *Candidatus* "K. Stuttgartiensis" has higher tolerance to nitrite, it is more active in low cell density cultures and it is less inhibited by phosphate compared to *Candidatus* "B. anammoxidans" (Egli et al., 2001).

The Anammox organisms resemble each other in the phylogenetic analyses of their 16S rRNA sequences, which show they form a monophyletic branch, which consists of five distinct genera with about 90% sequence similarity to each other, within the phylum *Planctomycetes* (Fig. 1.8).

As the rest of the species within the order *Planctomycetales*, they lack of peptidoglycan, an almost universal polymer found within the domain Bacteria. Instead, protein is the major constituent of their cell walls. Among the domain Bacteria, this lack of peptidoglycan is a characteristic shared only with the *Chlamydiae* and the cell-wall-free *Mycoplasms* (Lindsay et al., 2001).

As all known *Planctomycetes*, Anammox bacteria have an ultrastructure atypical for bacteria, with a compartmentalized cytoplasm (Fuerst, 2005). Electron microscopy analysis revealed that Anammox bacteria

have a separate specific membrane bound compartment: the anammoxosome. Hydroxylamine oxidoreductase (HAO) enzymes are present exclusively inside this anammoxosome, an organelle-like body that made up more than 30% of the cell volume (van Niftrik et al., 2004). This dedicated intracytoplasmic compartment has been found to be surrounded by a membrane nearly exclusively composed of unconventional membrane lipids: ladderane lipids (Sinninghe Damste et al., 2002). The structure of ladderane lipids is unique in nature, they have been found so far only in Anammox bacteria. These lipids are composed of pentacycloanammoxic acids, which contain five linearly concatenated cyclobutane rings (Mascitti and Corey, 2004). Due to the very slow metabolism of Anammox bacteria, a very dense and impermeable membrane is required to maintain concentration gradients during the Anammox reaction. Such a membrane also protects the cell from the toxic intermediates.

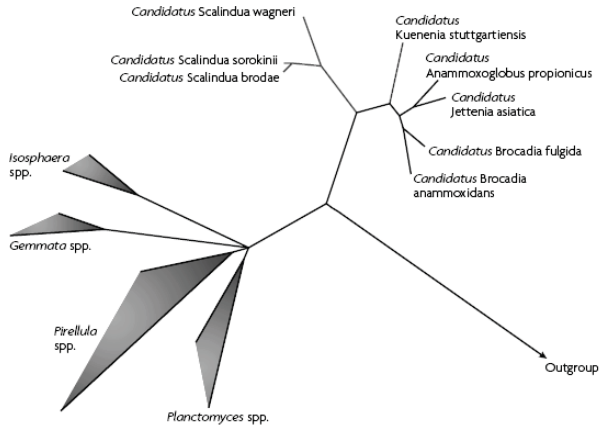

Figure 1.8. Anammox phylogenetic tree (Kuenen, 2008).

The genome of *Candidatus* "K. stuttgartiensis" has been sequenced (Strous et al., 2006). The genome data illuminate the evolutionary history of the Planctomycetes and Anammox bacteria in particular found to be related to a clade of intracellular parasites known as the *Chlamydiae*. Candidate genes responsible for ladderane biosynthesis and biological hydrazine metabolism were identified. Besides, an unexpected metabolic versatility was discovered, this versatility could justify why although slow and specialized, Anammox bacteria are global players in the biological nitrogen cycle (Arrigo, 2005).

1.5.5. Combination of processes

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

1.5.5.1. Nitrification-denitrification

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

Combining the equations obtained for the nitrification with those corresponding to the denitrification and taking into account the carbonate system equilibrium, the stoichiometry of the complete nitrogen removal by nitrification-denitrification with three different organic matter sources: methanol, acetate or acetic acid is as follows (Eq. 1.20 - 1.22).

Nitrification-denitrification over NO_3^-	
$NH_4^+ + 0.83\ CH_3OH + 2\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 4.17\ H_2O + 1.83\ CO_2$	(1.20)
$NH_4^+ + 0.625\ CH_3COO^- + 2\ O_2 + 0.375\ HCO_3^- \rightarrow 0.5\ N_2 + 3.125\ H_2O + 1.625\ CO_2$	(1.21)
$NH_4^+ + 0.625\ CH_3COOH + 2\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.75\ H_2O + 2.25\ CO_2$	(1.22)

1.5.5.2. Partial nitrification-Anammox

When the partial nitrification and the Anammox processes are combined certain considerations have to be taken into account. From the stoichiometry (Eq. 1.19) it can be inferred that Anammox bacteria need ammonium and nitrite in a ratio around 1:1.3. To reach this objective, half of the ammonium fed has to be converted in nitrite by AOB and therefore, the oxidation of nitrite to nitrate carried out by NOB has to be avoided.

Using this combined process of partial nitrification-Anammox, two alternatives are available to obtain autotrophic nitrogen removal. Using a two reactor configuration where half of the ammonium is oxidized to nitrite in the first reactor and a second Anammox reactor in anoxic conditions where ammonia and nitrite are removed producing a small amount of nitrate. Autotrophic nitrogen removal can also be achieved in a one reactor configuration where both populations: AOB and Anammox bacteria coexist under controlled aerobic conditions.

The global stoichiometry of the total process combining partial nitrification and Anammox is represented in Eq. 1.23.

$$NH_4^+ + 0.85\ O_2 + 1.11\ HCO_3^- \rightarrow 0.44\ N_2 + 0.11\ NO_3^- + 2.56\ H_2O + 1.11\ CO_2 \quad (1.23)$$

In comparison with the conventional nitrification-denitrification process, in the case of the Anammox based processes less oxygen is required and no organic matter must be present or added as external carbon source what makes this process suitable to treat wastewaters with low COD/N ratio, e.g., reject water. These processes are under research to be applied in WWTP for the removal of nitrogen.

1.6. Nitrogen removal treatment plants

1.6.1 Conventional WWTP configuration

Nowadays, activated sludge is the most used process for the biological treatment of domestic and industrial wastewaters. In most WWTP, wastewater is initially led to a stirred denitrifying basin with no air supply to avoid organic matter limitation in the denitrifying zone, and afterwards to a basin with aeration where nitrification occurs (Fig. 1.9). Nitrate containing wastewater from the aeration basin is recycled and mixed with the organic carbon rich wastewater entering the denitrifying zone. The nitrogen removal efficiency depends on the amount and presence of suitable organic substrates in the wastewater.

This strategy has several disadvantages such as: a) the simultaneous growth of autotrophic nitrifiers together with heterotrophic biomass which leads to relatively low nitrification rates, since heterotrophs grow much faster and b) the need of recirculation between both reactors. To ensure a sufficient nitrification rate, the

dissolved oxygen in the aerobic reactor has to be kept around 1-2 mg O_2 L^{-1}. The recirculation flow between both reactors permits to employ the alkalinity generated during the denitrification to control the pH in the nitrification unit. The percentage of nitrogen removal depends on the recirculation ratio.

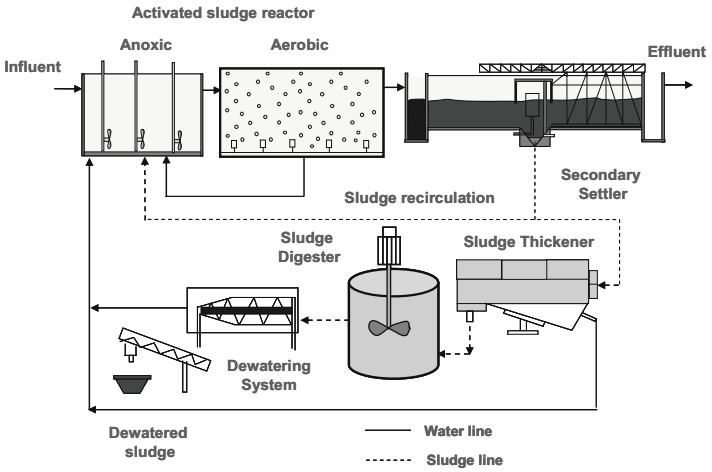

Figure 1.9. Scheme of a typical WWTP with sludge digester.

Nowadays, continuous-flow activated sludge systems almost always guarantee satisfactory performance with minimal supervision and maintenance for conventional wastewaters. There are several variations on the plug-flow scheme, depending on the treatment objectives. For example, processes such as Biodenitro, Biodenipho, UCT process and Bardenpho have been developed based on continuous design and involve different set-ups for achieving nitrification and/or aerobic-anoxic phosphorous removal (Metcalf and Eddy, 1995). Despite substantial practice and accumulated experience, more and more stringent effluent quality requirements, often imposing removal of nutrients together with organic carbon, and the increasing complexity of wastewaters seriously challenged their efficiency.

1.6.2. Main limitations of WWTP: management of reject water and excess sludge

Anaerobic digestion is becoming widely used to reduce sludge produced from the primary settling together with the excess of sludge from the biological reactor in WWTP with more than 40,000 I.E (Fig. 1.9). Anaerobic digesters are used to treat sewage under psycrophilic conditions with the COD removal efficiencies up to 90% if wastewater is pretreated (settling or coagulation-flocculation unit) (Aiyuk et al., 2006). The dewatered sludge produced can be burnt and the reject water highly concentrated in ammonium and poorly concentrated in biodegradable COD has to be treated since anaerobic digestion is only able to remove organic matter and, therefore, other processes should be applied to remove nutrients. This reject water generally accounts for only 2% of the total flow (Janus and van der Roest, 1997), but up to 25 % of the nitrogen load (Mulder et al., 2001; van Dongen et al., 2001). In case sludge from other wastewater treatment plants is treated at the same site, the contribution of reject water to the total nitrogen load of the WWTP can become even higher.

This reject water is normally recycled to the influent of the WWTP. In case that the main WWTP plant has been designed for carbon removal only, its aeration capacity is mostly insufficient to convert all ammonium

in the reject water. As the nitrification process is slow in comparison to carbon removal, the aerobic sludge retention time must also be sufficiently long, resulting in large reactor volumes. It is also possible that the WWTP possesses insufficient denitrification capacity, for instance when the plant has been designed for carbon removal and nitrification only. In this case, the anoxic volume should be enlarged (if possible) and/or an external carbon source has to be added (Siegrist, 1996). In Galicia, the main problem with the WWTP operation is the low concentration of COD due to the lack of stormwater main drain in the cities. This lack of selective drains and the high amounts of rainfall dilute the wastewater making the denitrification difficult in the anoxic tank due to a lack of biodegradable organic matter.

In the last years extensive research is being performed to improve nutrients removal in WWTP. Advanced processes were implemented in the last years even at real scale to overcome the problem of nitrogen removal due to the recirculation of reject water. The following alternatives are nowadays available:

- Bio-augmentation
- Partial nitrification-denitrification
- Partial nitrification-Anammox

1.6.3. Bio-augmentation

The bio-augmentation process consists of the separate treatment of reject water by the classical nitrification/denitrification process to remove ammonia from this stream. Nitrification/denitrification over nitrate for separate reject water treatment gains interest when an amount of surplus sludge from the reject water treatment, that contains both AOB and NOB, is recycled to the main wastewater treatment plant. In this way, the nitrification capacity of the latter is augmented. When designing or expanding a WWTP with bio-augmentation, a shorter SRT than for plants without bio-augmentation can be applied, resulting in smaller reactors to treat the same loads. In the side-stream process, complete nitrification to nitrate is required, as nitrification to nitrite only would result in only the growth of AOB, which could lead to nitrite accumulation in the main effluent of the plant (van Loosdrecht and Salem, 2006).

The BABE (Bio-Augmentation Batch Enhanced) process (Berends et al., 2005) can be operated as a one-reactor process as well as a two-reactor process. The one-reactor system is operated cyclically. In the first, aerobic phase, the reject water and the return sludge are fed and nitrification takes place. During the second, anoxic phase, denitrification takes place and the sludge settles. At the end of this phase, the reactor liquid is fed to the main wastewater treatment plant. The sludge has not settled completely at this moment, so inoculation with nitrifying sludge is performed. The two-reactor BABE process configuration (Salem et al., 2002) consists of an anoxic reactor, followed by an aerobic reactor. In the first (anoxic) reactor, the reject water stream is mixed with return sludge, which also serves as carbon source. When necessary, an external carbon source can be added as well. To supply the first reactor with nitrite and nitrate, recirculation takes place between both reactors.

The optimal SRT for bio-augmentation processes, maximizing the overall efficiency of the wastewater treatment plant as a whole, is determined by two opposite effects. On the one hand, a higher SRT results in a higher nitrification efficiency of the side-stream process. On the other hand, biomass decay also increases with increasing SRT. This results in less active biomass, which gives rise to a smaller augmentation effect on the activated sludge tanks (Berends et al., 2005).

The BABE® process was already established in several WWTP in the Netherlands, e.g., Hertogenbosch (350,000 I.E.) or Garmerwolde (300,000 I.E). In this city, the implementation of a one-reactor BABE process allowed reducing the ammonia concentration in the effluent of the WWTP from 13.3 to 5.2 mg $N\text{-}NH_4^+$ L^{-1}.

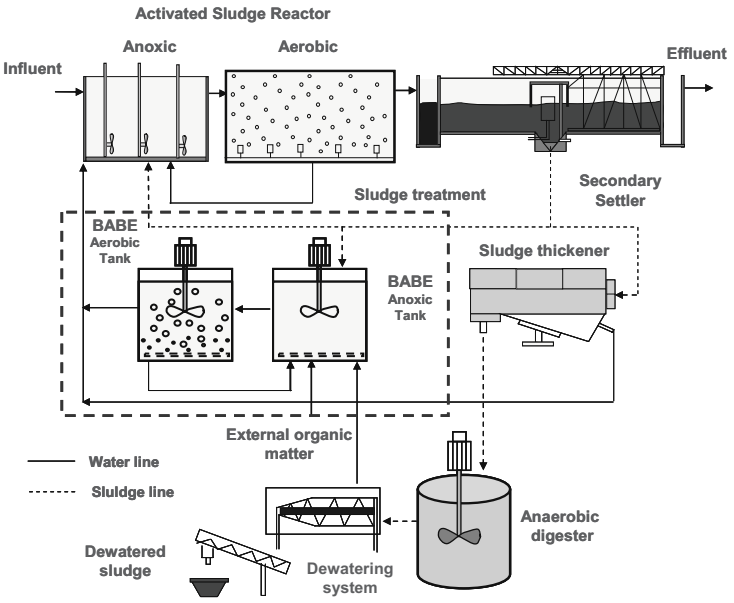

Figure 1.10. Two units BABE process.

1.6.4. Partial Nitrification/Denitrification

In this case, all the ammonium is partially nitrified, i.e., it is fully converted to nitrite and this nitrite is removed by heterotrophic denitrification. When comparing the stoichiometry of the whole nitrification-denitrification process over NO_3^- (Eq. 1.20 - 1.22) with the partial nitrification-denitrification over NO_2^- (1.24 - 1.26) it can be observed that the requirement of organic matter is 40% lower, the requirement of oxygen is 25% lower and less CO_2 is stripped. Moreover, lower sludge production is registered (Van Loosdrecht and Jetten, 1998); this fact makes this strategy interesting when working with wastewaters with low COD/N ratio where complete nitrification would not be achieved due to a lack of biodegradable organic carbon.

Nitrification-denitrification over NO_2^-	
$NH_4^+ + 0.5\ CH_3OH + 1.5\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.5\ H_2O + 1.5\ CO_2$	(1.24)
$NH_4^+ + 0.375\ CH_3COO^- + 1.5\ O_2 + 0.625\ HCO_3^- \rightarrow 0.5\ N_2 + 2.875\ H_2O + 1.375\ CO_2$	(1.25)
$NH_4^+ + 0.375\ CH_3COOH + 1.5\ O_2 + HCO_3^- \rightarrow 0.5\ N_2 + 3.25\ H_2O + 1.75\ CO_2$	(1.26)

However nitrogen removal of reject water with this process will be limited by organic matter availability since normally the organic matter present in the sludge liquor belongs to the slowly biodegradable fraction. Therefore to treat reject water, an external carbon source has to be added. Methanol (CH_3OH) is usually the substrate added due to its economic price.

The partial nitrification/denitrification strategy has been carried out in different configurations, e.g. the Sharon (Single reactor system for High-activity Ammonia Removal over Nitrite) process developed in 1997

(Hellinga et al., 1998). This process is carried out in a conventional CSTR with suspended biomass without sludge retention. At elevated temperatures (30 - 40 °C) and low SRT (in a CSTR, the HRT is equal to the SRT), AOB are selectively retained while the slower growing NOB, with growth rates lower than AOB at these temperatures, are washed out. Both nitrification and denitrification take place in the CSTR using intermittent aeration. Nitrogen removal efficiencies close to 100% can be achieved.

Economical balances demonstrated the saving up costs using the combined Sharon/denitrification processes to treat reject water in comparison to physicochemical processes or conventional nitrification/denitrification processes (Table 1.4).

Table 1.4. Estimation of costs for nitrogen removal in the sludge line for a WWTP of 500 000 I.E. (STOWA, 1996).

	Chemical sludge	**Biological sludge**	**Energetic Requirement**	**Cost** (Euro (kg N)$^{-1}$)
Physicochemical process				
Stripping with air	Yes	No	Normal	6.0
Stripping with steam	Yes	No	High	8.0
MAP process	Yes	No	Low	6.0
Nitrification/Denitrification				
Membrane reactor	No	Yes	High	2.8
Airlift reactor	No	Low	Normal	5.7
SHARON®/Denitrification	No	Low	Normal	1.5

Full-scale sludge liquor treatment with partial nitrification/denitrification in Sharon reactors has already been introduced at full scale in various WWTP (Table 1.5).

However, this principle for the selective enrichment of the ammonium oxidizing bacteria is not only restricted to CSTR operation; partial nitrification-denitrification was also successfully implemented in SBR reactors with careful control of the SRT (Fux et al., 2006; Dosta et al., 2007).

Table 1.5. Industrial scale Sharon reactors in operation (adapted from van Loosdrecht and Salem, 2006).

WWTP	**Capacity** (I.E.)	**N load** (kg N d^{-1})	**Year of start-up**
Utrecht	400 000	900	1997
Rotterdam	470 000	830	1999
Zwolle	150 000	540	2000
Beverwijk	320 000	1 200	2004
Garmerwolde	300 000	700	2004
Den Haag	1 100 000	1.200	2005
New York	3 000 000	5.500	2007

1.6.5. Autotrophic nitrogen removal: Partial nitrification/Anammox

The discovery of Anammox processes allows nowadays the performance of the autotrophic nitrogen removal. The application of the combination partial nitrification/Anammox to treat reject water (Table 1.6) infers several advantages:

- Saving up aeration costs. The combination of partial nitrification/Anammox minimizes the aeration requirements since only half of the ammonium has to be oxidized to nitrite. As aforementioned, only 0.85 moles of O_2 are needed to treat 1 mole of NH_4^+ (Eq. 1.23) whereas 1.5 moles of oxygen per mol of NH_4^+ (Eq. 1.26) are needed when using the combination partial nitrification-heterotrophic-denitrification and 2 moles of O_2 per mol of NH_4^+ (Eq. 1.22) in the case of using the conventional nitrification-denitrification pathway.
- No organic matter is needed which makes this strategy interesting to treat wastewater without biodegradable organic carbon source or with low COD/N ratio.
- In the case of rejection effluents from sludge digesters, about half of the ammonium in the liquor can be converted without any pH control, and this causes the depletion of the alkalinity of the water. This leads to a pH drop and avoids further nitrification (Jetten et al., 2002). Therefore, in this case, the pH is auto regulated and it is not necessary the addition of chemicals what makes economically viable this strategy. As aforementioned, with the alkalinity available in reject water, only half of the ammonium is oxidized and therefore no pH correction would be necessary with the subsequent reagents saving.
- Less sludge is produced in comparison to the nitrification/denitrification strategy and the partial nitrification/denitrification. The fact that less sludge is produced allows saving money since the treatment of the sludge generated represents up to 50-60% of the WWTP budget.
- Less CO_2 is stripped.
- Besides the lower CO_2 release to the atmosphere when using Anammox based processes, the produced amount of other gases which contribute to the greenhouse effect, e.g., N_2O is also lower. If nitrification or denitrification processes are not fully accomplished, gases like NO or N_2O can be released into the atmosphere contributing to the greenhouse effect (Mulder, 2003; Campos et al., 2009). Under unfavourable conditions, like a high load and a low COD/N ratio, the emission of N_2O can contribute to 10% in the total N-balance (Mulder, 2003). The gas N_2O is about 320-fold more efficient than CO_2 in contributing to global warming. It is reported by Mulder (2003), that this emission occurs largely in agriculture (50%), fertiliser production (6%) and wastewater treatment (21%).
- Finally, Siegrist et al., (2008) reported that, with the application of partial-nitrification/Anammox, WWTP are coming closer to energy autarky. With the introduction of separate digester liquid treatment the denitrification efficiency in the biological main treatment can be reduced without reduction of the overall nitrogen removal of the whole plant. This allows increasing the HRT of the primary clarifier to improve biogas production in the anaerobic digester and significantly reduce the aeration energy for BOD removal and nitrification.

Table 1.6. Theoretical comparison of biological N-removal processes on stoichiometric basis, for streams with equimolar amounts of ammonium and bicarbonate (Volcke et al., 2004).

Processes[a]	O_2 consumption (kg O_2 (kg N)$^{-1}$)	COD consumption (kg COD (kg N)$^{-1}$)	CO_2 emission (kg CO_2 (kg N)$^{-1}$)	Sludge production[b] (kg VSS (kg N)$^{-1}$)
Nitrification-Denitrification (including bio-augmentation)	4.57	2.86	5.76	1 - 1.2
Partial Nitrification-Denitrification (over NO_2^-)	3.43	1.71	4.72	0.8 - 0.9
Partial Nitrification-Anammox	1.71	0	3.14	< 0.1

[a]Methanol was used as carbon source, the values were obtained from Eq. 1.20, 1.23 and 1.24.
[b]Mulder, 2003.

As aforementioned, the critical aspect of the autotrophic nitrogen removal is the development of a critic mass of Anammox bacteria due to their slow growth rate. To overcome this drawback, good biomass retention capacity of the reactors and the optimisation of conditions favouring the Anammox process are mandatory. Two different configurations can be applied to combine partial-nitrification and Anammox processes: a two reactor configuration and a one reactor configuration.

1.6.5.1. Two reactors configuration

In this case, partial nitrification and Anammox processes are carried out in two separated units. The first reactor is operated under aerobic conditions to convert half of the ammonium to nitrite, whereas the second reactor is operated under anaerobic conditions to obtain autotrophic denitrification which is performed by Anammox bacteria. The most common system to reach partial nitrification is the Sharon reactor.

The Sharon reactor is operated only under aerobic conditions and at temperatures over 30 ºC controlling the HRT to wash out NOB but keeping AOB inside the reactor. The control and operation of the Sharon reactor has been studied by Van Hulle et al., (2007) and Volcke et al., (2006) who reported the kinetic constants of AOB and NOB in a Sharon reactor and designed a robust control system (DO and pH were the control parameters) to optimize the ammonium/nitrite ratio in the effluent. The Sharon reactor also demonstrated being suitable to treat the effluent of an anaerobic digester treating the wastewater generated in a fish canning industry with high concentrations of salts (Mosquera-Corral et al., 2005a).

Gali et al., (2007) developed partial nitrification in a SBR. They used the same concept: of high temperature of operation and control of the SRT and compared the results with a Sharon configuration. They concluded that the kinetic and stoichiometric parameters of the ammonium oxidation process were similar for both configurations. Biomass retention in the SBR would result in smaller reactor volume for the given influent ammonium concentrations to be converted. The SHARON process showed however, a better stability against starvation periods or changing loads than this SBR.

The second step, the Anammox process can be carried out in different reactors, e.g., UASB (Upflow Anaerobic Sludge Blanket) or IC (Internal Circulating) reactors similar to the ones used in the anaerobic processes. At laboratory scale, the SBR is widely used due to its flexibility of operation and easy control, e.g., Dapena-Mora et al., (2004b) showed that the SBR is a suitable system to grow Anammox biomass in the form of granular sludge.

Full scale reactors with the Sharon-Anammox configuration have already been implemented in the Netherlands. The low growth of Anammox microorganisms delayed the start-up of the process but the design capacity of nitrogen removal (750 kg N d^{-1}) was finally achieved after 3.5 years (Table 1.7).

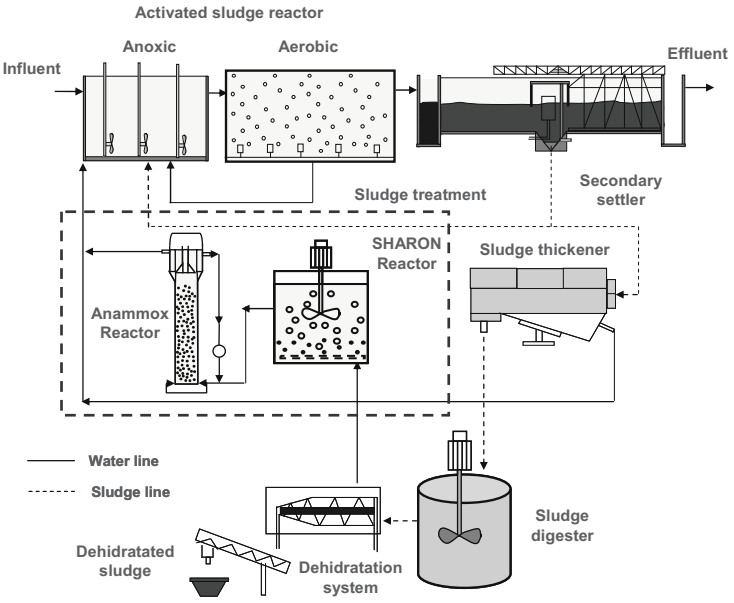

Figure 1.11. Sharon-Anammox process to treat reject water.

1.6.5.1. One reactor configuration

The partial nitrification and the Anammox processes can also be carried out together in a single reactor. This combined process has been called different names, CANON: Completely Autotrophic Nitrogen removal Over Nitrite process (Third et al., 2001), OLAND: Oxygen-Limited Autotrophic Nitrification-Denitrification (Kuai and Verstraete, 1998), deammonification (Hippen et al., 1997; Helmer et al., 2001) and SNAP: Single-stage Nitrogen removal using Anammox and Partial nitritation (Furukawa et al., 2006). Once the single unit option is chosen the process occurs only under controlled aerated conditions. Under oxygen-limited conditions a co-culture of aerobic and anaerobic ammonium-oxidizing bacteria can be established in a single unit.

With the development of biofilms, aerobic and anoxic zones can be present within the biofilm due to the gradients of oxygen generated with oxygen consumption by aerobic microorganisms. This will allow the development of AOB in the aerobic layers and Anammox bacteria in the anoxic ones (Fig. 1.12). In those systems, NOB growth has to be avoided since the oxidation of nitrite to nitrate is not desired. NOB compete for oxygen with the aerobic AOB and for nitrite with Anammox bacteria, and thus its growth (and subsequent nitrate production) can be prevented, e.g., controlling the DO concentration in the bulk liquid (Rosenwinkel and Cornelius, 2005).

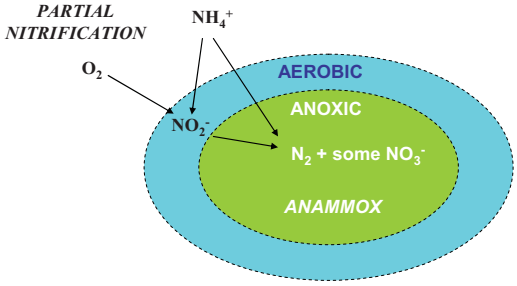

Figure 1.12. CANON process in biomass aggregates.

The one reactor configuration has been developed at full scale to treat reject water in different reactors and biofilm configurations but all of them applied to effluents with temperatures ranging between 30-40 ºC since this is the optimum range of temperatures for Anammox bacteria. Wett (2006, 2007) developed the autotrophic nitrogen removal process working with aggregates: the DEMON® system which used pH to control the two steps of the nitrogen removal. This process was first implemented in Austria and then in Switzerland. The availability of inoculum and the experience learned reduced the start-up time from 2.5 years to 2 months (Table 1.7). The autotrophic nitrogen removal was also developed at full scale in biofilms grown on Kaldness rings in MBBR (Moving Bed Biofilm Reactor) in Hattingen (Rosenwinkel and Cornelius, 2005) and Sweden (personal communication) (Table 1.7).

Table 1.7. Anammox plants at industrial scale (adapted from Abma et al., 2007 and Wett, 2007).

Project	Application	Volume[a] (m^3)	Capacity achieved (kg N d^{-1})	Start-up time
Waterboard Hollandse Delta, The Netherlands (2 units)	Municipal (reject water)	72	750	3.5 year
Strass, Austria (1 unit)	Municipal (reject water)	500	350	2.5 year
IWL, The Netherlands (2 units)	Tannery	100	150[b]	1 year
Waterstromen, The Netherlands (1 unit)	Potato processing	600	700[b]	6 months
Himmerfjärdsverket[c], Sweden (1 unit)	Municipal (reject water)	700	240	6 months
Glanerland, Switzerland (1 unit)	Municipal (reject water)	400	250	2 months
Semiconductor Plant, Japan (2 units)	Semiconductor	58	220	2 months

[a] For two units systems the volume corresponds to the Anammox unit.
[b] No more nitrogen available.
[c] Personal communication.

1.6.6. Comparison of the available technologies to treat reject water

The bio-augmentation, partial-denitrification and partial nitrification-Anammox technologies can be applied to optimize the nitrogen removal in WWTP. The selection of one of the strategies will depend on the specific limitations in the nitrogen removal of each plant according to the decision tree represented in Fig. 1.13. The first decision concerns whether the limiting step in the nitrogen removal in the main plant is the nitrification

or the denitrification. In case the nitrification or denitrification are limited by the volume of the aerobic or the anoxic tank, the bio-augmentation is the recommended technology.

However, if the aeration or the organic matter content is limiting the nitrogen removal, the "nitrite" route processes are recommended. The ammonium counter-ion plays an important role and will determine the most suitable technology to be applied. In most WWTP this counter ion will be the bicarbonate ion which works as pH buffer in the nitrification process and the recommended technology would be the partial nitrification-Anammox.

Finally, if the sludge is dried the counter-ion will be the acetate ion; in this case, the partial nitrification-denitrification will be the most suitable technology. Besides these aspects, the start-up time, the risk of failure, the flexibility of the process, etc. will also determine the decision of which technology to implement.

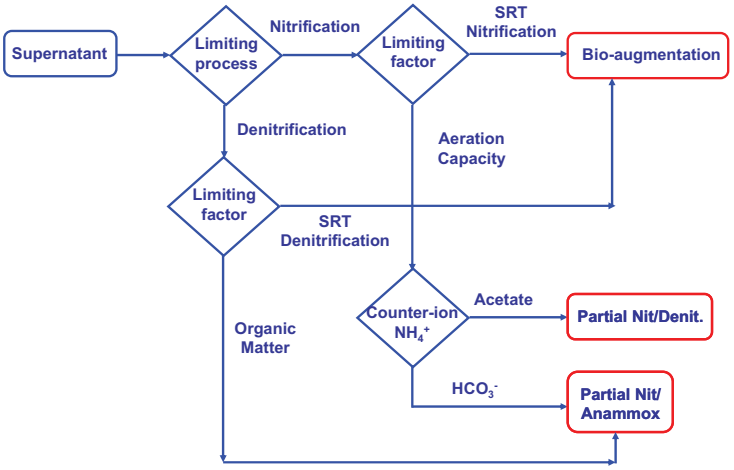

Figure 1.13. Diagram of selection of the best technology to treat reject water.

1.7. Systems based on aerobic granulation

As aforementioned the main drawback of autotrophic microorganisms is their slow growth rate in comparison to heterotrophic bacteria. This makes the start-up of biological reactors based on autotrophic bacteria difficult. To overcome this drawback, the withdrawal of microorganisms has to be avoided and therefore, the SRT in the bioreactors has to be high enough to accumulate the slowest microorganisms, e.g., Anammox biomass. In the last years, aerobic granulation arose as a new technology to accumulate high biomass concentrations and consists of the development of aggregates that grow without the need of carrier material as a special kind of biofilm. Biofilms are ubiquitous life forms consisting of microorganisms embedded in a matrix of biological origin called extracellular polymeric substances.

Aerobic granular sludge consists of compact and dense microbial aggregates with a spherical outer shape, which represent a particular or special case of biofilm development (Liu and Tay, 2004). The aerobic formation of such structures is linked with the stressing operating conditions imposed for its development, resulting in a self microbial adhesion process, which initiates the granule formation (Liu and Tay, 2004). The interest in developing aerobic granular sludge systems is mainly related to the compactness of their design in comparison to conventional activated sludge systems due to the higher biomass concentration achieved inside

the biological unit and to the lower settling size required. The application of this kind of systems can also contribute to the reduction of sludge production by three ways:

a) Reduction of the amount of sludge generated: Tay et al., (2001) 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.

b) Reduction of sludge volume: during the treatment of sludge generated most of the processes applied are focused on reducing its volume by decreasing its water content. Granules have denser and more compact structures than flocs which caused directly a lower sludge volume. On the other hand, during granulation cells increase their hydrofobicity from 50 to 80% which could favour dewatering processes (Tay et al., 2001).

c) Improvement of anaerobic sludge digestion: the biodegradability of aerobic granules is also higher than flocs due to its high content of polyhydroxybutyrate (Fang et al., 2009).

Recent research showed that it is possible to grow Granular sludge in Sequencing Batch Reactors (GSBR) at large dissolved oxygen concentrations using either synthetic wastewater (Beun et al., 1999; Mosquera-Corral et al., 2005b) or industrial effluents (Arrojo et al., 2004; Tsuneda et al., 2006). The operational sequence of GSBR is specially designed to enhance the formation of fast settling granules in which two different zones can be distinguish: (i) the outer aerobic shell, in which heterotrophic and autotrophic microorganisms will remove COD and oxidize ammonium into nitrate, respectively, and (ii) the inner anoxic core in which denitrification can take place. In addition, the phosphate removal is possible due to the use of anaerobic/aerobic phases in the sequential operation, which enable the development of specific biomass, the so called polyphosphate accumulating organisms (PAO).

The aim of this thesis is linked to the application of the autotrophic nitrogen removal. The granulation concept is interesting since by having favourable conditions for granulation, stable Anammox population would develop in the inner core of aerobic granules. The consumption of DO in the external layers will allow the presence of internal anoxic layers where oxygen is not available.

1.7.1. Modelling of granular systems

The complexity of modelling biofilm systems such as granular biomass thrives in the need of combining diffusion and reaction kinetics. A wide range of parameters influence the characteristics and performance of biofilms or granules. Therefore mathematical models represent an important and fundamental tool to know in detail the processes developed in the granules. Furthermore they can provide a solid foundation for design and operation without the time consumption and materials expense of the experimental approach. Anyway models, to be valuable, must be calibrated and validated with experimental data.

For the conventional floc-based activated sludge systems, the Activated Sludge Model (ASM) established by the International Water Association (IWA), provides a consistent framework for the description of biological processes. The ASM can be used as a basis to develop the aerobic granule models but introducing several modifications. The IWA developed four Activated Sludge Models, the ASM1, ASM2, ASM2d and ASM3 (Henze et al., 2000). The ASM1 is the structure and platform for further development. The ASM1 allows the simulation of organic matter removal and biological nitrification and denitrification (N-removal) in activated sludge systems. The ASM2 is an extension of the ASM1 and includes biological phosphorous

removal processes, and the ASM2d includes denitrifying PAOs. The ASM3 is a new modelling platform based on recent developed knowledge of the activated sludge processes and includes the possibility of following up the concentrations of internal storage compounds. The decay process from ASM1 is replaced by an endogenous respiration process in ASM3, a more realistic approach under a microbiological point of view (Van Loosdrecht and Jetten, 1998).

The matrix structure and organization of ASM models makes an easy integration of these models into several simulation programs feasible: AQUASIM, WEST, Plan-It STOAT… and also the developement of the model using mathematical tools as MATLAB.

In the ASM, biomass is assumed in suspension in the bulk liquid. However, this approach is not suitable when bacteria grow forming colonies that arise in biofilms, aggregates or aerobic granules. The aggregation of bacteria creates a separation between the bulk liquid and the biological media (Fig. 1.14). It can be assumed that the bulk liquid is well mixed and therefore homogeneous, however, in the bacterial phase biological processes and substrate diffusion velocities provoke concentration gradients of substrates: DO, COD, ammonium, nitrate, nitrite, phosphates… Those concentration gradients are influenced by many factors, e.g., diffusion coefficients, conversion rates, granule size, biomass spatial distribution, density, etc. There are several influences between every factor, thus the effect of separate factors cannot be studied experimentally, and the simulation models can give more insight at a microscopic scale by solving differential equations.

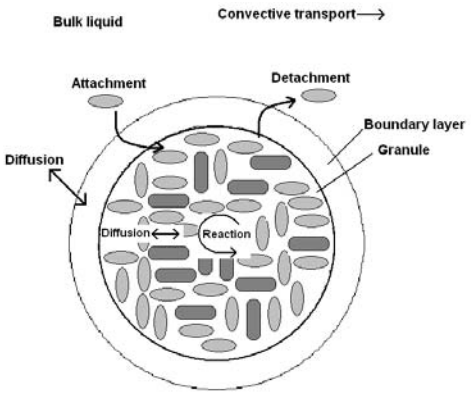

Figure 1.14. Relevant processes considered in an aerobic granule.

Lübken et al., (2005) researched if the model ASM3 could be used as a first simplification to simulate nutrient removal with aerobic granular sludge in a SBR. The model proved to be capable of describing the performance of a lab-scale SBR reactor concerning COD, ammonium and nitrate in the bulk liquid. However, for describing the processes inside of a granule, biofilm processes should be included in the model.

The mathematical modelling of biofilms has gained increasing interest in the last decade and a wide range of biofilm models of different complexity applied to different types of benchmark problems has been developed. Aerobic granules can be viewed as a special type of biofilm but without carriers for the biofilm attachment. For this reason models of aerobic granular systems have been based on biofilm models. Modelling of biofilm or granular structures is possible using one dimensional models (1-D) or multidimensional models (2-D and 3-D). One dimensional models are simpler and therefore require less computational effort. Nevertheless, in 1-D models several assumptions are made. It is assumed that the substrates gradients are some orders of

magnitude higher in the perpendicular direction to the attachment surface than in the parallel plane to it. Important characteristics derived from the dynamics of biofilm structure must be taken into account once assumed 1-D transport. Some examples of properties that must be explicitly defined are: external and internal mass transfer coefficients, changes in pore volume and motility of bacterial species inside the biofilm matrix (Xavier et al., 2005).

Extensive research is being performed to model granular systems. Beun et al., (2001) described the COD and N removal in a granular sludge batch reactor using a 1-D model implemented in AQUASIM. These authors showed that nitrification, denitrification and COD removal can occur simultaneously in a granular sludge SBR. Su and Yu, (2006) established a generalized model for aerobic granular SBR taking into account the removal of nitrogen and organic compounds, reactor hydrodynamics, oxygen transfer and substrates diffusion. Recently Ni et al., (2008) used a modification of the ASM3 model to describe the simultaneous autotrophic and heterotrophic growth in aerobic granular SBR, implementing the model in AQUASIM. De Kreuk et al., (2007) introduced the removal of phosphate in addition to the removal of COD and nitrogen as a process in the model. They used a 1-D model to study the influence of different parameters (oxygen concentration, temperature, granule diameter, sludge loading rate and cycle configuration) on the granular SBR operation.

The development of multidimensional (2-D or 3-D) models offers more potential to predict local compositions of particulate and dissolved variables. Several approaches to multidimensional modelling of biofilm can be found in the literature (Xavier et al., 2005). In general 2-D and 3-D biofilm models could be divided in two classes according to the way of describing the biomass: discrete individual particles or a continuum body. Discrete particle models are the most suitable for extrapolation to granular biomass. Individual-based models (Ibm) are, discrete particle models, widely applied to study effects of spatially multidimensional gradients in biofilms. Xavier et al., (2007) presented a multiscale model of a granular sludge batch reactor describing the complex dynamics of populations and nutrient removal.

There is an extensive research to perform in the field of nitrogen removal from the point of view of the involved processes themselves if autotrophic processes are required and of the diffusion limitations in case of working with biofilms or granules. Information obtained from modelled results is of great interest in order to reduce the number of experiments to be performed.

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Chapter 2

Materials and Methods

Summary

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

From the conventional chemical parameters measured in the liquid phase the Total and Soluble Chemical Oxygen Demand and in the solid phase the Total, Inorganic and Volatile Suspended Solids (TSS, ISS and VSS) and Settling Velocity were determined following Standard Methods (APHA-AWWA-WPCF, 1998). These are therefore not further described in this chapter. Nitrogen in the form of ammonium (NH_4^+), Total Organic and Inorganic Carbon (TOC, IC) and several inorganic anions (NO_2^-, NO_3^-, Cl^-; PO_4^{3-} and SO_4^{2-}) have been measured by analytical procedures optimised in our laboratories and these are thus described in detail throughout this chapter. The biomass was characterised also by means of parameters such as sludge volume index, granules density and techniques of digital image analysis, electronic microscopy and stereomicroscope.

Identification of the different populations present in the biomass samples was carried out by Fluorescent In Situ Hybridization (FISH). To obtain the distribution of bacteria, the FISH technique was applied to cryosectioned granules which were cut in slices. Confocal laser microscopy was used to obtain images of the bacterial stratification along the granules.

Finally, the two microelectrodes used in this study to determine oxygen and nitrite inside the granular biomass are described The specific analytical methods used in a single part of the work are described in the corresponding chapter, as well as the corresponding experimental set-ups.

2.1. Liquid phase

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

2.1.1. 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 Chemical Oxygen Demand (COD), but does not provide the same information. Unlike COD, TOC is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen and hydrogen, and inorganics that can contribute to the oxygen demand measured by COD (APHA-AWWA-WPCF, 1998). To determine the quantity of organically bound carbon, the organic molecules must be broken down and converted to a single carbon molecular form that can be measured quantitatively. The TOC concentration was determined by a Shimadzu analyzer (TOC-5000) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. The instrument is connected to an automated sampler (Shimadzu, ASI-5000-S). The TC concentrations are determined from the amount of CO_2 produced during the combustion of the sample at 680 °C, using platinum immobilised over alumina spheres as catalyst. The IC concentrations are obtained from the CO_2 produced in the chemical decomposition of the sample with H_3PO_4 (25%) at room temperature. The CO_2 produced is optically measured with a nondispersive infrarred analyzer (NDIR) after being cooled and dried. High purity air is used as carrier gas with a flow of 150 mL min^{-1}. A curve comprising 4 calibration points in the range of 0 to 1 g C L^{-1}, using potassium phthalate as standard for TC and a mixture of sodium carbonate and bicarbonate (Na_2CO_3/$NaHCO_3$, 3:4 w/w) for IC, is used for the quantification (Fig. 2.1).

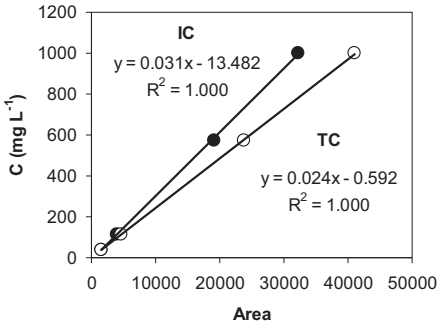

Figure 2.1. Calibration curve to determine TC and IC concentrations.

2.1.2. Ammonium nitrogen

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

2.1.2.1. Reagents preparation

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

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

2.1.2.2. Determination procedure

Place 2.5 mL of sample (diluted if necessary to get a maximum concentration of 1 mg NH_4^+-N L^{-1}) and add, 1.0 and 1.5 mL of solution 1 and 2, respectively. After waiting 45 min at room temperature, the concentration of NH_4^+-N is measured in a spectrophotometer at 635 nm. The quantification is done with a 5-7 points calibration curve in the range of 0-1 mg NH_4^+-N L^{-1}, using NH_4Cl as standard (Fig. 2.2).

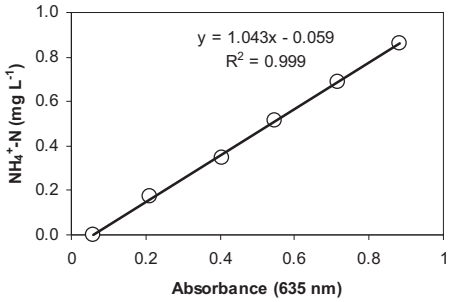

Figure 2.2. Calibration curve for ammonium concentration determination.

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

Nitrite (NO_2^-), nitrate (NO_3^-), chloride (Cl^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) are determined simultaneously by Waters Capillary Ion Analyzer (CIA). A solution of sodium cromate (0.005 mol L^{-1}) is used as electrolyte (Vilas-Cruz et al., 1994). An electro-osmotic modifier (50 mL L^{-1}) CIA-Pak™ OFM Anion BT Waters (Ewing et al., 1989) is also added to this solution. The sample is forced to migrate through a capillary (melting silica covered with poliimida, 60 cm long and 75 μm of internal diameter) kept at 25 °C by the application of an electric current. Depending on the ratio charge/mass of the ion, the migrating time is different. A hydrostatic injection (10 cm height for 30 seconds) and an indirect detection (UV, 254 nm, 20 kV, 16-22 μA) are used. A number of 4 to 6 calibration points for each ion in the range of 3-100 mg L^{-1} are daily used for the quantification of the samples (Fig. 2.3).

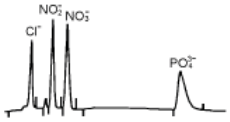

Figure 2.3. Typical electropherogram

2.1.4. Other control parameters

2.1.4.1. pH

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

2.1.4.2. Dissolved oxygen (DO)

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

2.1.4.3. Determination of the oxygen gas-liquid transfer coefficient (k_La)

An experimental estimation of the oxygen gas-liquid transfer coefficient (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 SBR in the absence of biomass after the reestablishment of the aeration beginning with concentrations of oxygen close to zero. The mass balance was applied to oxygen according to Eq. 2.1.

$$\frac{dC_{O2}}{dt} = k_L a \cdot \left(C^*_{O2} - C_{O2}\right) \quad (2.1)$$

After integration Eq. 2.2 is obtained.

$$\ln\left(\frac{C^*_{O2} - C^{t=0}_{O2}}{C^*_{O2} - C_{O2}}\right) = k_L a \cdot t \quad (2.2)$$

The k_La coefficient was estimated according to Eq. 2.2, being C_{O2}^* the saturation concentration at the experimental temperature, $C_{O2}^{t=0}$ the DO concentration at the beginning of the experiment and C_{O2} the measured DO concentration in the bulk liquid.

2.2. Biomass characterisation

2.2.1. Sludge Volumetric Index

The Sludge Volumetric Index (SVI) determination is defined in the Standard Methods for the Treatment of Water and Wastewater (APHA-AWWA-WPCF, 1998) as the volume in millilitres occupied by 1 g of a suspension after 30 min settling. However, as suggested at the "*1st IWA-Workshop Aerobic Granular Sludge*" (de Kreuk et al., 2005) and by Schwarzenbeck et al. (2004) another parameter, the SVI_5 (SVI after 5 minutes of settling) was used in all the chapters instead of SVI_{30} (SVI after 30 minutes of settling) since it is more representative for granular biomass. A low SVI_{30} does not necessarily imply sludge granulation and viceversa. Nevertheless a granular sludge bed does consolidate much faster, i.e., the terminal SVI_{30} is already reached after 5 minutes of settling.

2.2.2. 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. Then, a known amount of liquid is removed from the sample. A known volume of a dextran blue solution (1 g L^{-1}) is added to a representative sample (and known amount) of granular sludge, in a volume ratio of about 1:1. The mixture is gently mixed, and subsequently the granules are allowed to settle. A known amount of the liquid above the settled granules is removed and a sample is taken from it. This fraction and the original dextran blue solution are analyzed by a spectrophotometer at 620 nm. Subsequently the volume occupied by the biomass in the reactor sample is calculated, since dextran blue only binds to water and not to biomass. Measuring also the dry weight of the reactor sample (APHA-AWWA-WPCF, 1998) the density of the granules as g biomass per L of granules can be calculated.

The density is calculated with Eq. 2.3 and V_L with Eq. 2.4.

$$\text{Density} = \frac{V_{initial} \times VSS}{V_{biomass}} \quad (2.3)$$

being: VSS: Volatile Suspended Solids (g L^{-1})

$V_{initial} = P_2 - P_1$ (L)

$V_{biomass} = P_4 - P_1 - V_L$ (L)

P_1: test tube weight

P_2: weight of the test tube with sample

P_3: weight of the test tube with sample after removal of liquid

P_4: weight of the test tube after dextran blue addition

$$V_L = \frac{A_{initial} \times V_{dextran}}{A_{final}} \quad (2.4)$$

being: $A_{initial}$: Absorbance of the dextran blue solution (1 g L^{-1})

A_{final}: Absorbance of the sample

$V_{dextran}$: is calculated as the difference between P_4 and P_3

2.2.3. Average diameter of the granules

Changes in morphology of the granules were followed by image analysis (Tijhuis et al., 1994). Images of the granular sludge were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (SMZ-2T, Nikon and Stemi 2000-C, Zeiss, respectively). For digital image analysis the programme Image ProPlus® was used. The procedure followed is represented in Fig. 2.4 and is as follows:

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

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

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

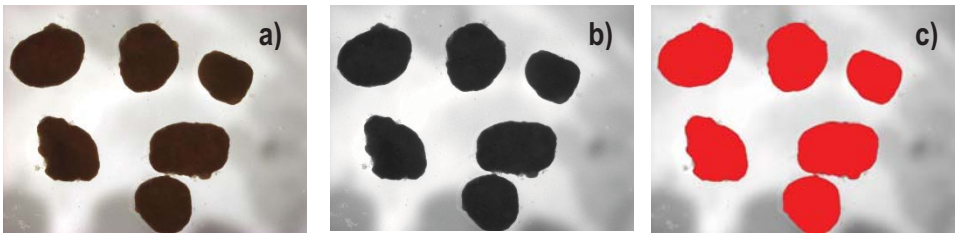

Figure 2.4. a) Original image of a sample of granules b) Image of the granules converted to black and white c) Area recognized by the software in red once the threshold levels are defined by the user.

The average diameter obtained from the programme corresponded to the mean feret diameter of the granules. The feret diameter is calculated as an average value from the shortest and the longest measured segment in the granule (Fig. 2.5).

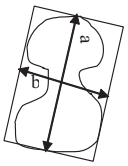

Figure 2.5. Longest and shortest segments in a granule to estimate the feret diameter of a granule.

2.2.4. Specific Anammox activity assays

The batch assays used to estimate the Anammox activity were performed according to the methodology described by Dapena-Mora et al. (2007). Completely closed vials with a total volume of 38 mL with 25 mL of liquid volume were used to perform the Anammox batch assays (Fig. 2.6).

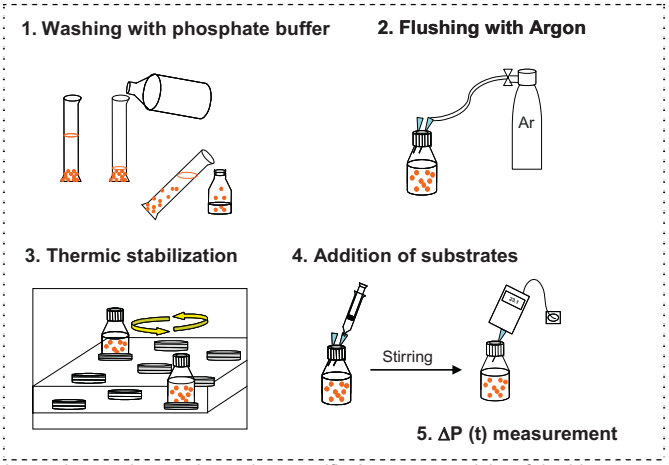

Figure 2.6. Experimental procedure to determine specific Anammox activity of the biomass.

Biomass concentration at the beginning of the experiment was fixed around 1.0 g VSS L^{-1}. Before the beginning of the batch test the biomass was washed three times with phosphate buffer (0.143 g KH_2PO_4 L^{-1} and 0.747 g K_2HPO_4 L^{-1}). The pH value was fixed at 7.8 and the temperature was fixed at a value T depending on the conditions to be analyzed. Gas and liquid phases were purged with argon gas to remove O_2. The vials were placed in a thermostatic shaker, at 150 rpm and the temperature T until stable conditions were reached. Initial concentrations of substrates were 70 mg NH_4^+-N L^{-1} and 70 mg NO_2^--N L^{-1}. The production of N_2 was determined in the gas phase as the increment of pressure in the headspace of the vials, measured by means of a pressure transducer device.

Maximum Specific Anammox Activity (SAA) was estimated from the maximum slope of the curve described by the cumulative N_2 production along the time and related to the biomass concentration in the vials. The N_2 gas production rate (moles N_2 min^{-1}) was calculated from the maximum slope of the curve describing the pressure increase in the vial along time (α) (atm min^{-1}) (Eq. 2.5).

$$\frac{dN_2}{dt} = \alpha \frac{V_G}{R \cdot T} \tag{2.5}$$

being V_G the volume of the gaseous phase (L), R the ideal gas coefficient (atm L mol^{-1} K^{-1}) and T the temperature (K).

The SAA (g N_2-N g VSS^{-1} d^{-1}) is calculated from the N_2 gas production rate and the biomass concentration in the vial (g VSS L^{-1}):

$$SAA = \frac{dN_2/dt}{X \cdot V_L} \frac{28\ g\ N}{mol\ N_2} \frac{1440\ min}{d} \tag{2.6}$$

being V_L the volume of the liquid phase (L).

Since the values of the affinity constant of the anammox bacteria for ammonium and nitrite are lower than 10 μM and 5 μM, respectively (Strous et al., 1999), it can be considered that the activity measured is the maximum activity for the range of nitrite and ammonium concentrations used.

2.2.5. Electron Microscopy and Micro-analysis

Morphological studies of the biomass were performed with a scan electron microscope (Digital SEM Leica 440 at 20 kV) controlled with a computer system and with a magnification capacity ranging from 15 to 290000 folds. The sludge sample was washed three times for 10 minutes with phosphate buffer 0.05 N at pH 7.4 and subsequently fixed with a solution of glutaraldehyde 3% in phosphate buffer overnight. After fixation the sample was dehydrated using ethanol solutions with increasing ethanol concentrations (30, 50, 70 and 100%). Later the sample was shaded with gold and observed under the scan electron microscope.

To investigate the elemental composition of the granules a micro-analysis was carried out. The instrument used was the SEM LEO-435VP with a system of micro-analysis (EDX) at voltages varying in the range of 5 kV, 20 kV and 30 kV. A typical count of the different atoms detected in a sample is represented in Fig. 2.7.

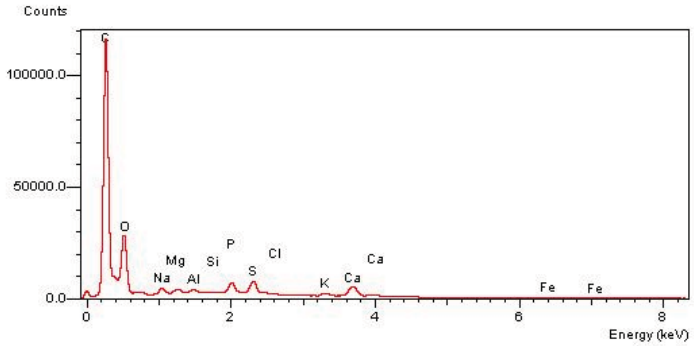

Figure 2.7. Representation of the different atoms present in a sample of biomass detected in a micro-analysis.

2.3. Microbiological determinations

2.3.1. Identification of bacteria populations by FISH

The abundance of the different populations of microorganisms present in the sludge samples of the reactors was researched by Fluorescent In Situ Hybridization (FISH). With this technique specific regions in 23S or 16S rRNA are detected with fluorescently labelled probes. If the corresponding domain, phylum, genus or species is present, the probe hybridizes to the targeted sequence and can later be detected microscopically. According to Amann et al. (1995) a typical FISH protocol includes four steps (Fig. 2.8): the fixation and permeabilization of the sample; hybridization of the targeted sequence to the probe; washing steps to remove unbound probe; and the detection of labelled cells by microscopy or flow cytometry. This protocol must be applied to disrupted biomass; therefore, the granules must be disintegrated before starting the procedure. To achieve the granular biomass breakage, biomass is sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher).

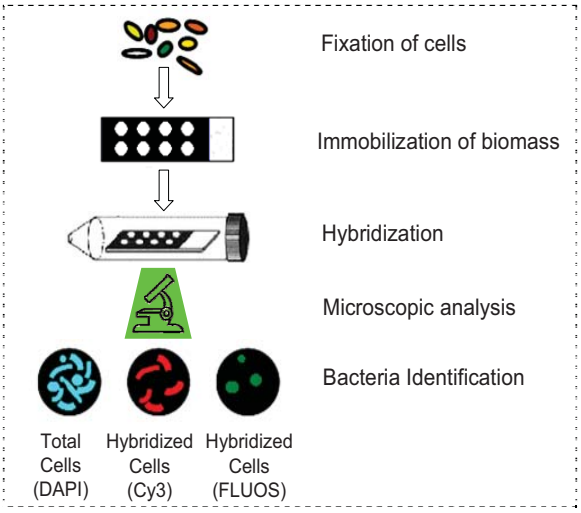

Figure 2.8. Different steps of the typical FISH protocol

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

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

The fluorochromes used to detect the hybridized rRNA were FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccinimide ester) and Cy3 (indocarbocyanine). To visualize all cells in a sample the stain 4,6-diamidino-2-phenylindole (DAPI) was used. Its application can provide insight into the existence of archaeo-bacteria and eukaryotes, like e.g. protozoa. For analysis of the slides an epifluorescence microscope (Axioskop 2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics) was used. The schematic tree reflecting the different probes applied in this study indicating the bacteria detected by each probe are shown in Fig. 2.9. The probes applied in this study are listed and detailed in Table 2.2.

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

For further discussion it has to be kept in mind that samples can never be 100% representative. Thus the fact that no bacteria of a certain kind were present in the sample can always be attributed to unrepresentative sampling as well. Still it was tried to keep this error small.

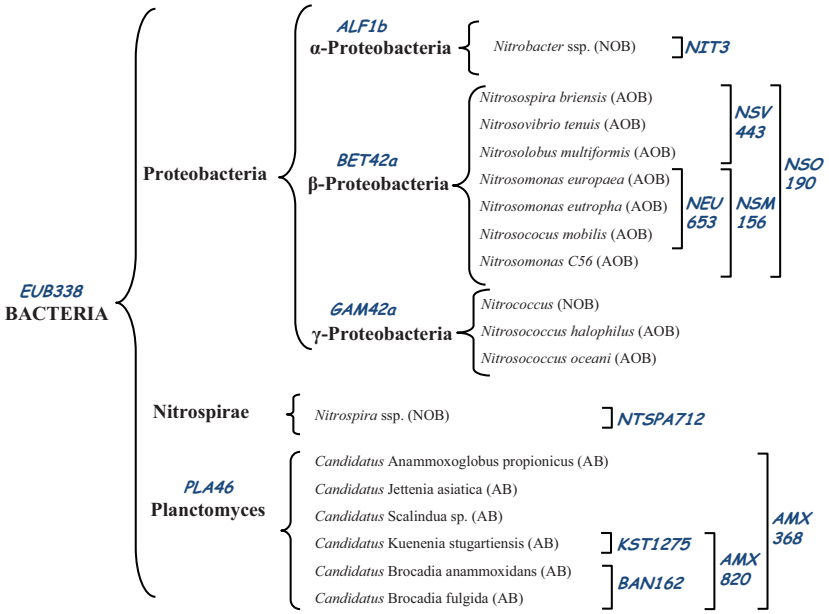

Figure 2.9. Different probes applied and the main bacteria detected by each probe (AOB: ammonium-oxidizing bacteria, NOB: nitrite-oxidizing bacteria and AB: Anammox bacteria).

Table 2.2. Probes used for fluorescent in situ hybridisation and the formamide (FA) concentration used during hybridization.

Probe	Target site 16S	Probe sequence (5'→3')	% FA	Target organisms	Ref.[a]
EUB 338	338-355	GCT GCC TCC CGT AGG AGT	0-50	Bacteria domain	[1]
EUB 338 II	338-355	GCA GCC ACC CGT AGG TGT	0-50	Planctomycetales	[2]
EUB 338 III	338-355	GCT GCC ACC CGT AGG TGT	0-50	Verrucomicrobiales	[2]
ALF1B	19-35	CGT TCG YTC TGA GCC AG	20	α-proteobacteria, some δ-proteobacteria, Spirochaetes	[3]
BET42a	1027-1043	GCC TTC CCA CTT CGT TT	35	β-proteobacteria	[3]
GAM42a	1027-1043	GCC TTC CCA CAT CGT TT	35	γ-proteobacteria	[3]
NEU653	653-670	CCC CTC TGC TGC ACT CTA	40	Most halophilic and halotolerant Nitrosomonas spp.	[4]
Competitor		TTC CAT CCC CCT CTG CCG			
Nso190	189-207	CGA TCC CCT GCT TTT CTC C	55	Ammonio-oxidizing-β-*Proteobacteria*	[5]
Nsm156	156-174	TAT TAG CAC ATC TTT CGA T	5	Nitrosomonas spp., Nitrosococcus mobilis	[5]
NIT3	1035-1052	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.	[6]
Competitor		CCT GTG CTC CAG GCT CCG			
Ntspa712	712-732	CGC CTT CGC CAC CGG CCT TCC	35	Most members of phylum *Nitrospira*	[7]
Competitor		CGC CTT CGC CAC CGG GTT CC			
PLA46	46-63	GAC TTG CAT GCC TAA TCC	20	Planctomycetes	[8]
Amx820	820-841	AAA ACC CCT CTA CTT AGT GCC C	35	*Candidatus* "Brocadia anammoxidans"	[9]
Kst157	157-174	GTT CCG ATT GCT CGA AAC	25	*Candidatus* Kuenenia stuttgartiensis	[9]
Ban162	162-179	CGG TAG CCC CAA TTG CTT	40	*Candidatus* Brocadia anammoxidans	[9]
Pae997	997-1014	TCT GGA AAG TTC TCA GCA	0	*Pseudomonas* spp.	[10]
PAR1244	1244-1262	GGA TTA ACC CAC TGT CAC C	20	*Paracoccus*	[11]

[a][1] Amann et al., (1990) [2] Daims et al., (1999) [3] Manz et al., (1992) [4] Wagner et al., (1995) [5] Mobarry et al., (1996) [6] Wagner et al., (1996) [7] Daims et al., (2001) [8] Neef et al., (1998) [9] Schmid et al., (2001). [10] Amann et al., (1996) [11] Neef et al., (1996).

2.3.2. Cryosectioning and confocal microscopy

In order to determine the stratification of bacteria along the granule, some aggregates were frozen and cut in slices. Entire granules were embedded in OCT reagent (Tissue-Tek; Miles, Ind.) prior to their cryosectioning at -35 °C. Slides with a thickness ranging form 14 to 25 μm were cut at -16 °C, and these single sections were placed on the surface of poly-L-lysine coated microscopic slides. Hybridization was performed with the protocol aforementioned. The used probes for in situ hybridization were 5' labelled with the fluorochromes FLUOS, Cy3 and Cy5 (Indodicarbocyanine). After in situ hybridization cells were stained with DAPI (0.5 μg mL^{-1}) for 10 minutes. A TCS-SP2 confocal laser scanning microscope (Leica, Germany), equipped with HeNe laser for the detection of the fluorochromes Cy3 and Cy5 and Ar laser for the detection of the fluorochrome FLUOS, was used to analyze the sliced samples.

Quantification of the bacterial population was based on the procedure published by Crocetti et al., (2002). The quantification was performed by comparison of the positive area obtained with a probe with the area corresponding to the control: DAPI or EUBmix (a mixture of EUB338, EUB338 II and EUB338 III). The digital image analysis software Image ProPlus® was used to quantify the areas. When analyzing images with different probes, the software Leica Confocal Software Lite® was used to isolate each fluorochrome which corresponded to a specific probe. The area corresponding to the fluorescence of each FISH probe was obtained as the area of all pixels above a manually determined minimum pixel intensity to exclude empty spaces and background fluorescence (the sequence of analysis is represented in Fig. 2.10).

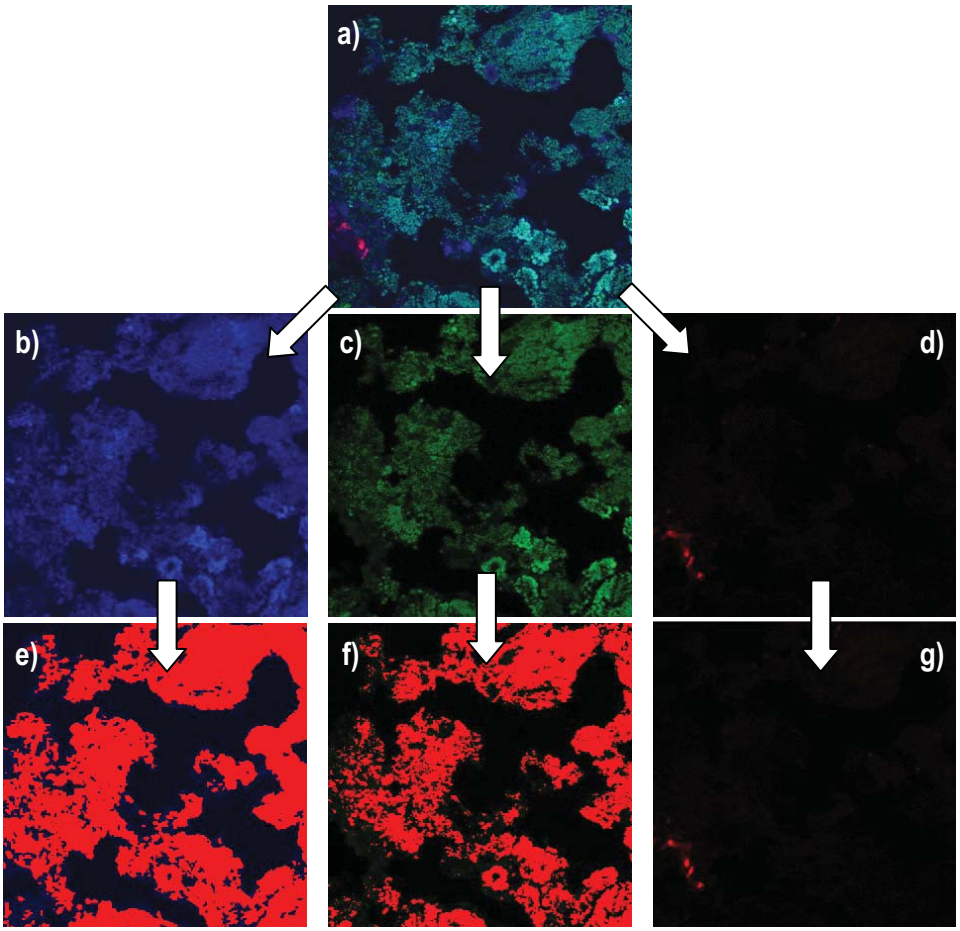

Figure 2.10. a) Confocal image from a triple hybridization with the probes EUBmix (blue), NEU653 (green) and Amx820 (red). b) c) d) Images of each individual probe separated with the Leica Software. e) f) g) Images of the area detected for each probe by the Image Pro Plus software.

2.4. Microelectrodes

By the introduction of few micrometer thick microsensors for analysis of a wide range of chemical species in microbial ecology, it became possible to study on a small scale the chemical gradients and

metabolism in stratified microbial communities (e.g., Revsbech and Jorgensen, 1986). Microelectrodes are widely used to determine concentrations gradients of different compounds inside biofilms, core sediments, soils, etc. due to their small sizes. Due to the availability of very thin sensors, it is possible to perform experiments that cause minimal disturbance to the analyzed medium. Two different microelectrodes were used in this thesis: an electrochemical oxygen microsensor and a nitrite microbiosensor.

2.4.1. O_2 microelectrode

A Clark-type O_2 microsensor (Fig. 2.11) equipped with a guard cathode was used for microscale analysis of DO; a detailed description of the functioning of this electrochemical microelectrode is given in Revsbech, (1989). The O_2 microelectrode is an amperometric sensor; which detects the current caused by electrochemical reactions of the analyte at the sensor tip. A potential difference between the sensing electrode and the reference drives the reaction, and the current, measured by a sensitive picoammeter, is proportional to the analyte concentration (Schramm, 2003). These microsensors are characterized by absence of interferences, perfectly linear calibration curves, fast response times and low effects on the signal (<1–2%) from changes in stirring or diffusivity of the external medium. The sensors used in this study were constructed with tip diameters of 10 µm and they had 90% response time lower than 1 s. The very fast response time makes it possible to record detailed concentration profiles over distances of millimeters within a few minutes. The oxygen microsensor was calibrated at two points, the zero was established introducing the microelectrode in an anoxic alkaline ascorbic acid solution and the saturation was obtained both with an air saturated solution or with pure oxygen saturated solution depending on the DO concentration to be analyzed. The O_2 microelectrodes used in this work were built by the technical assistant Preben Sorensen, a member of the microbiology department of Aarhus University (Denmark).

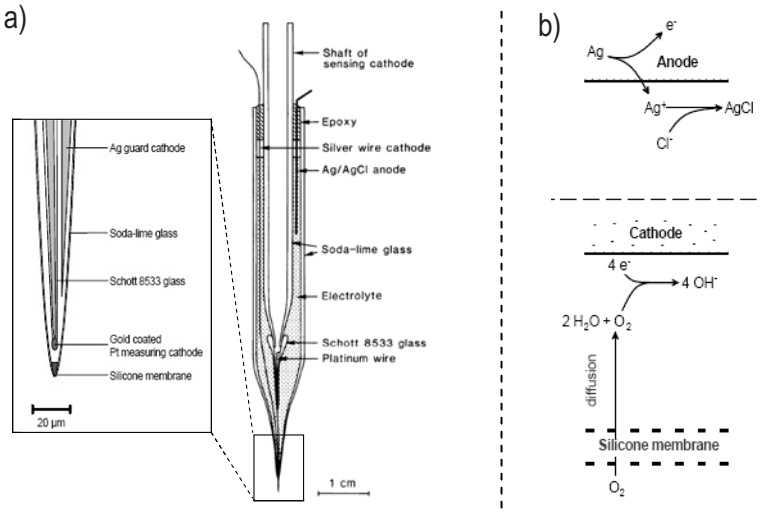

Figure 2.11. a) Clark-type O_2 microelectrode (adapted from Revsbech, 1989) b) Schematic of the O_2 measuring principle in the microelectrode (from Kuhl and Revsbech, 2001).

2.4.2. NO_2^- microelectrode

For some sensors a biological reaction precedes the electrochemical reaction, and these sensors are labeled as microbiosensors. The NO_2^- microbiosensor was based on a combination of biological reactions catalyzed by bacteria and an electrochemical N_2O microsensor (Fig. 2.12). The NO_2^- microbiosensor (a detailed description is available in Nielsen et al., 2004) consisted of an immobilized pure culture of *Stenotrophomonas nitritireducens*, which specifically reduced NO_2^- to N_2O (they lack the enzyme N_2O reductase), coupled to a Clark type N_2O microsensor (Andersen et al., 2001). A medium reservoir supplied the culture with all essential growth constituents except the electron acceptor. NO_2^- diffused freely through the ion-permeable membrane into the bacterial biomass of the reaction chamber, where it is denitrified to N_2O and subsequently reduced to N_2 at the cathode of the N_2O sensor. By placing the bacteria in a capillary in front of an electrochemical N_2O microsensor, the signal of the N_2O electrode was proportional to the concentration of NO_2^-. The 90% response time was 30-50 s, slower than the O_2 microsensor but also making possible to perform concentration profiles over distances of millimeters within a few hours.

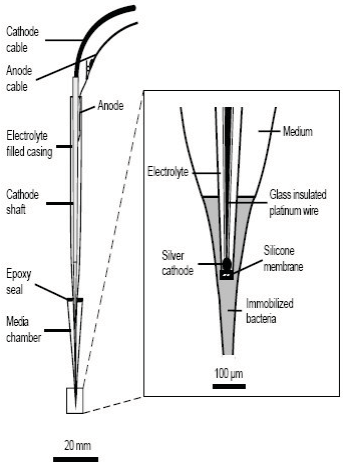

Figure 2.12. NO_2^- microbiosensor (Kuhl and Revsbech, 2001).

The microelectrodes were calibrated before every profile determination. The NO_2^- microbiosensors were calibrated in a vial containing NO_2^--free water with approximately the same ionic strength and temperature as the environment where the measurements were to be performed. A calibration curve was obtained by plotting the current values from the N_2O electrode against various NO_2^- concentrations obtained by repeated addition of NO_2^- from a concentrated stock solution (Fig. 2.13). Tungstate was added inside the biosensor in order to measure NO_2^- concentrations higher than 7 mg N L^{-1}. The tungstate increased the stability of the microsensor and slowed down the nitrite consumption rate of the bacteria (Nielsen et al., 2005).

The lifetimes of microscale sensors containing immobilized bacteria are from days to weeks. The NO_2^- microelectrodes used in this work were built by the Professor N.P. Revsbech, member of the microbiology department of Aarhus University (Denmark).

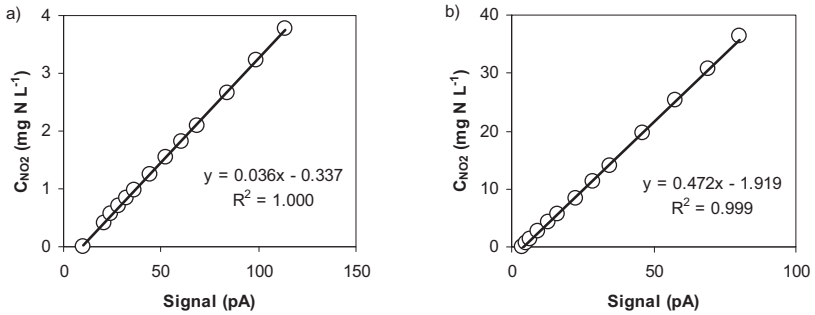

Figure 2.13. Calibration curves of two NO_2^- microelectrode a) without addition of tungstate b) with addition of tungstate at different signal intensities.

2.4.3. Experimental setup

A setup for measuring O_2 and NO_2^- in the granules is shown schematically in Fig. 2.14. The microelectrode is held by a micromanipulator, which is used to introduce the microelectrode tip into the granule. The current in the circuit is measured by a very sensitive ammeter with a range down to 1 pA (10^{-12} A). The amplified signal is then recorded on a strip-chart recorder.

The granules were fixed on a mesh grid by a needle at the bottom of the aerated cell. Granules were kept for 1 h inside the cell as a pre-incubation period to create pseudo steady state conditions, the air was provided by an aeration pump. Concentration profiles were recorded by introducing the sensors into the granules at different depth positions using a manual micromanipulator. A dissection microscope was used to visually estimate the position of the granule/water interface by visual observation. For each granule and experimental condition tested several microprofiles were measured.

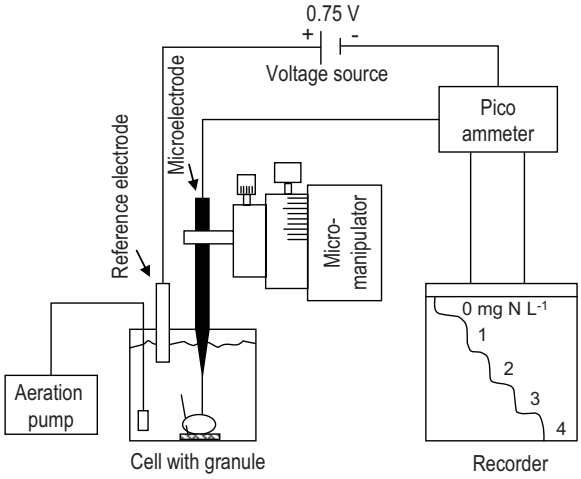

Figure 2.14. Circuit used for the obtaining of profile measurements with microelectrodes (Adapted from Revsbech and Jorgensen, 1986).

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Chapter 3

Nitrifying granular systems: a suitable technology to obtain stable partial nitrification at room temperature[1]

Summary

The operation of nitrifying biomass as biofilms or granules allows the treatment of higher ammonia loads compared to the conventional activated sludge systems, due to the large amounts of biomass accumulated inside the reactors. In the present work, a sequencing batch reactor (SBR) containing nitrifying granules with an average diameter around 3 mm was operated for 880 d at room temperature (18-24 ºC). The obtained granules presented high settling velocities around 100 m h^{-1} and low sludge volumetric indexes of 30 mL (g VSS)$^{-1}$ which indicated their good settling properties. When nitrogen loading rates (NLR) of 0.4 g NH_4^+-N L^{-1} d^{-1} were fed, complete nitrification to nitrate was reached. The increase of the NLR to 0.8 g NH_4^+-N L^{-1} d^{-1} caused a stable accumulation of nitrite with only 20% of nitrate production even by keeping the dissolved oxygen (DO) concentration in the bulk liquid at 8 mg O_2 L^{-1}. Batch assays performed in a range from 2 to 30 mg O_2 L^{-1} of DO showed that partial nitrification of 50% of the ammonium to nitrite was reached by fixing the DO concentration in the range from 2.0 to 3.5 mg O_2 L^{-1} in the bulk liquid. The total surface of the granules to bulk liquid ratio was low and ranged from 58 to 216 m^2 m^{-3} and therefore the oxygen mass transfer through the interface "bulk liquid−granule surface" was limiting. This oxygen transfer limitation allowed reaching stable partial nitrification at room temperature. For more than one year, an effluent with a mean NO_2^-/NH_4^+ molar ratio close to 1.0, suitable to feed a subsequent Anammox reactor was obtained. A maximal nitrite production of 1 g NO_2^--N L^{-1} d^{-1} was reached without nitrite oxidation to nitrate. The bacteria populations in the granules belonged mainly to the genus *Nitrosomonas* and were place in the external layer of the granules of 100 μm.

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[1]**Vázquez-Padín J.R., Figueroa M., Campos J.L., Mosquera-Corral A. and Méndez R**. (2010) Nitrifying granular systems: a suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology* **74**: 174-186.

3.1. Introduction

Excess of nitrogen content in dumped wastewater causes oxygen depletion and enhances eutrophization of the receiving natural streams. Nitrogen is commonly present in wastewater in the form of ammonium, which can be removed by means of different treatment processes classified in two categories: physicochemical and biological processes. The use of the conventional biological nitrification-denitrification processes for nitrogen removal in wastewater treatment plants is recommended due to their lower costs compared to the physicochemical methods (van Dongen et al., 2001).

In order to apply this combined nitrification-denitrification processes several aspects have to be taken into account. Firstly, the nitrification is generally the limiting process; therefore, it is important to promote the accumulation of large concentrations of nitrifying biomass inside the reactors. This purpose can be achieved using biofilm systems where large retention times for nitrifiers are obtained. Nowadays the operation of aerobic granular biomass systems is under study as a new approach to the biofilm systems. The development and operation of nitrifying granular sludge has already been reported (Van Benthum et al., 1996; Campos et al., 2000; Tsuneda et al., 2003; Mosquera-Corral et al., 2005a). Secondly, in order to achieve complete denitrification a certain amount of organic carbon source has to be available in the treated effluent. In many cases the addition of external organic matter is necessary which causes an increase of treatment costs.

In order to overcome this drawbacks several alternatives are under study based on the use of the so called “nitrite route”, which is a shortcut in the nitrogen cycle based on the oxidation of ammonia to nitrite and the further reduction of the latter to nitrogen gas. This route can be carried out according to two alternatives:

1) Combining the partial nitrification of ammonium to nitrite and the subsequent heterotrophic denitrification using an organic carbon source as electron donor. This alternative involves the saving of aeration and addition of electron donor source costs in 25% and 40%, respectively, compared to the conventional nitrification-denitrification processes (Van Loosdrecht and Jetten, 1998).

2) Combining the partial nitrification of half of the ammonia contained in the wastewater followed by the Anammox (anaerobic ammonia oxidation) process. The Anammox process consists of the oxidation of ammonia using nitrite as electron acceptor in the absence of dissolved oxygen (Strous et al., 1999). This alternative is recommended for the treatment of wastewaters without biodegradable organic carbon source. This option allows achieving the largest saving costs in terms of aeration (40%) and electron donor source supply (100%) respectively, compared to the conventional nitrification-denitrification processes (Van Loosdrecht and Jetten, 1998).

Common to both alternatives is the use of a partial nitrification unit, where the ammonia is oxidized to nitrite, with efficiencies of 100% and 50%, corresponding to options 1) and 2) respectively. As the nitrification process consists of two serial reactions carried out by the ammonia oxidizing bacteria (AOB) and the nitrite oxidizing bacteria (NOB) respectively, nitrite accumulation is expected to occur when the oxidation rate of ammonia is faster than that of nitrite. When trying to obtain partial nitrification the NOB activity has to be avoided in order to restrict the ammonia oxidation to nitrite. The AOB and NOB are two phylogenetically unrelated groups characterized by different growth rates affected in a different way by parameters like temperature, pH, dissolved oxygen (DO), etc. These differences can be exploited to uncouple both reaction rates and to outcompete NOB. With this objective several strategies based on the modification of these parameters have been used to reach partial nitrification:

1) Temperature: The fact that the activation energy of the ammonia oxidation step is higher than that of the nitrite oxidation step provokes that an increase of temperature involves an increase on the ammonia oxidation rate larger than that experienced by the nitrite oxidation rate. In practice, this means that the maximum specific growth rate of the AOB will be higher than that of NOB at temperatures above 25 ºC. In fact this is the basis of the SHARON technology, which consists of a chemostate reactor operated at a hydraulic retention time (HRT) of 1 day and 30 ºC to favour the growth of the AOB and to washout the NOB (Hellinga et al., 1998; Mosquera-Corral et al., 2005b).

2) Free ammonia and free nitrous acid inhibition: It is known that *Nitrosomonas* are inhibited by concentrations of free ammonia over 10 mg NH_3-N L^{-1} while *Nitrobacter* are inhibited by values of only 0.1-1.0 mg NH_3-N L^{-1} (Anthonisen et al., 1976). Therefore, partial nitrification could be obtained by maintaining levels of free ammonia in the reactor which caused only inhibition of the NOB population (Carrera et al., 2004).

3) DO concentration: Stable nitrite accumulation has been obtained in oxygen limited biofilm reactors by keeping the DO concentration in the bulk liquid to values around 1-2 mg O_2 L^{-1} (Garrido et al., 1997; Bernet et al., 2005). This effect is due to the higher oxygen half saturation constant of NOB compared to AOB (Carrera et al., 2004; Blackburne et al., 2008a). Values of ammonium oxidation rate (AOR) as high as 5 g N L^{-1} d^{-1} were obtained by Garrido et al. (1997) in a BAS (Biofilm Airlift Suspension Reactor), containing 30 g VSS L^{-1} of biomass, due to the large biofilm surface to bulk liquid volume ratio (3700 m^2 m^{-3}). Nitrite accumulation was registered in this reactor due to a decrease in the k_La caused by the accumulation of large biomass concentrations in the reactor which diminished the oxygen transfer limitation from gas to liquid.

3.2. Objectives

● The development and long term operation of the partial nitrification process at temperatures around 20 ºC in a sequencing batch reactor (SBR) were nitrifying biomass in the form of aerobic granules was grown.

● The maintenance of a nitrite production efficiency of 50%, via the control of DO concentration, to generate an effluent suitable to feed an Anammox reactor operated at low temperature.

3.3. Materials and methods

3.3.1. Reactor description

A sequencing batch reactor (SBR) with a working volume of 1.5 L was used (Fig. 3.1). Dimensions of the unit were: height of 46.5 cm and inner diameter of 8.5 cm, the height to the diameter ratio being 5.5. The exchange volume was fixed at 50%. Oxygen was supplied by using air spargers to promote the formation of small bubbles to guarantee the complete mixture and the oxygen supply. The gas flow was kept constant at 1.7 L min^{-1} (corresponding to a superficial air velocity of 0.5 cm s^{-1}). DO concentrations in the continuous operation of the reactor ranged from 2.0 to 8.5 mg O_2 L^{-1}. In the experiments where values lower than 8 mg O_2 L^{-1} were required, a mixture of N_2 gas with air was used maintaining the total flux of gas constant. A set of two peristaltic pumps was used to introduce the feeding solution (on top of the reactor) and to discharge the effluent (at medium height in the column reactor), respectively. A programmable logic controller Siemens model S7-224CPU controlled the actuations of the pumps and valves, and regulated the different periods of the operational cycle.

3.3.2. Operational conditions

The granules used in this work were initially grown treating wastewater from a dairy industry (Arrojo et al., 2004). Then, the composition of the influent was changed to stepwise decrease the COD/N ratio in the feeding to 0 (Mosquera-Corral et al., 2005a). At the beginning of this experiment, the SBR contained nitrifying biomass in the form of granules with a biomass concentration of 1.5 g VSS L^{-1}. The reactor was able to oxidize to nitrate the ammonium fed at a nitrogen loading rate (NLR) of 0.4 g N L^{-1} d^{-1}.

The composition of the autotrophic feeding medium was, in g L^{-1}: 0.38 – 1.52 NH_4Cl (which corresponds to 0.1 – 0.4 g N L^{-1}), 2.5 $NaHCO_3$, 0.092 K_2HPO_4, 0.036 KH_2PO_4, 0.049 $MgSO_4$, 0.019 KCl and 0.5 mL L^{-1} of a trace solution. The composition of the trace solution was in g L^{-1}: 1.50 $FeCl_3 \cdot 6\ H_2O$, 0.15 H_3BO_3, 0.15 $CoCl_2 \cdot 6\ H_2O$, 0.12 $MnCl_2 \cdot 4\ H_2O$, 0.12 $ZnSO_4 \cdot 7\ H_2O$, 0.06 $NaMoO_4 \cdot 2\ H_2O$, 0.03 $CuSO_4 \cdot 5\ H_2O$ and 0.03 KI.

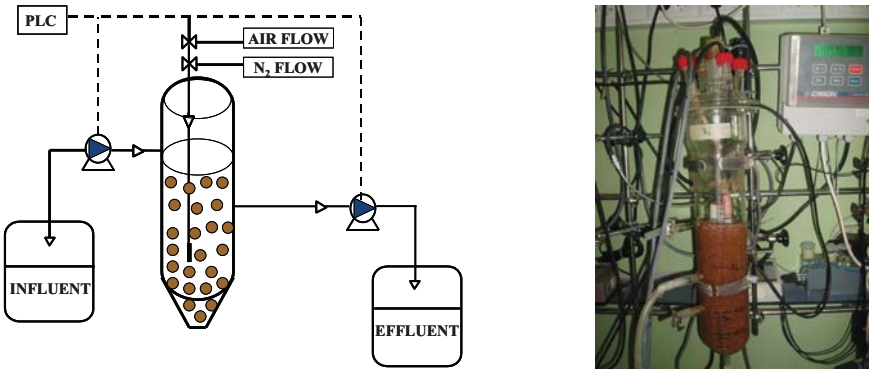
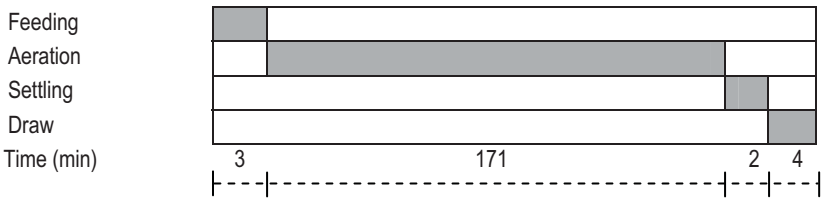


Figure 3.1. Scheme and photograph of the SBR.

The SBR was operated in cycles of 3 h, which were adjusted according to two different organized distributions of the periods (Fig. 3.2).

a) Cycle A: Discontinuous operation



b) Cycle B: Semicontinuous operation

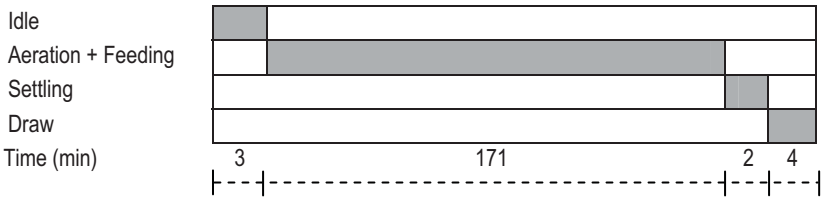


Figure 3.2. Distribution of the operational cycles.

During the reactor operation using the Cycle A distribution the volume of the reactor was kept constant at 1.5 L during the entire aerobic reaction period. When the reactor was operated according to Cycle B distribution, the reactor volume varied during the aerobic reaction period from 0.75 to 1.50 L by feeding the reactor with an influent flowrate of 4.4 mL min^{-1}. The reactor was operated during the whole operational period at room temperature (18 - 24 ºC). The pH was not controlled and ranged between 7.0 and 8.5.

The main changes in the operational conditions of the SBR are summarised in Table 3.1. On day 400, corresponding to the beginning of period IV, 2 g VSS of granules form another reactor, treating a synthetic wastewater with a COD/N ratio of 5 and performing organic matter oxidation and complete nitrification, were inoculated.

Table 3.1. Main operational conditions in the different stages of the SBR reactor.

Stage	Days	Cycle distribution	NLR (g N L^{-1} d^{-1})	DO (mg O_2 L^{-1})
I	0 – 61	A	0.4	7.5 – 8.5
II	62 – 142		0.8	
III	143 – 399	B		
IV[a]	400 – 504			
V	505 – 692			2.0 – 3.5
VI	693 – 880		0.8 – 1.6	

[a]Addition of granules from another granular SBR.

3.3.3. Analytical methods

The pH, ammonium, volatile suspended solids (VSS) and sludge volumetric index (SVI_5) were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Nitrite and nitrate concentrations were determined by capillary electrophoresis (Vilas-Cruz et al., 1994). DO concentration was measured with a dissolved oxygen probe (AQUALITYC, model OXI-921) connected to a meter (M-Design Instruments TM-3659). 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) provided with a digital camera (Coolsnap, Roper Sicientific Photometrics). For the digital image analysis the programme Image Pro Plus was used. The density of the granular biomass was determined using the method described by Beun et al., (2002).

Nitrifying populations in the biomass samples were identified by the Fluorescence in situ hybridization (FISH) technique. Granules from the reactor were collected, kept in their aggregated form or disaggregated and fixed with 4% paraformaldehyde (Amann et al., 1995). Entire granules were embedded in OCT reagent (Tissue-Tek; Miles, Ind.) prior to their cryosectioning at -35 °C. Slides with a thickness of 25 μm were cut at -16 °C, and these single sections were placed on the surface of poly-L-lysine coated microscopic slides. Hybridization was performed at 46 ºC for 90 minutes adjusting the formamide concentrations at the percentages shown in Table 3.2. The used probes for in situ hybridization were 5' labelled with the dyes FLUOS or Cy3. Fluorescence signals of disaggregated samples were recorded with an Axioskop 2 epifluorescence microscope (Zeiss, Germany) while a TCS-SP2 confocal laser scanning microscope (Leica, Germany), equipped with a HeNe laser for detection of Cy3 and an Ar ion laser for detection of FLUOS, was used with the sliced samples. For further information regarding the analytical methods is provided in Chapter 2.

Table 3.2. Targeted organisms and the corresponding formamide (FA) percentages for the used oligonucleotide probes.

Probe[a]	Probe sequence (5'→3')	FA (%)	Targeted organisms
EUB338	GCT GCC TCC CGT AGG AGT	0-50	*Bacteria* domain
EUB338 II	GCA GCC ACC CGT AGG TGT	0-50	Planctomycetales
EUB338 III	GCT GCC ACC CGT AGG TGT	0-50	Verrucomicrobiales
ALF1b	CGT TCG Y(C/T)TC TGA GCC AG	20	α-proteobacteria, some δ-proteobacteria, spirochaetes
BET42a[b]	GCC TTC CCA CTT CGT TT	35	β- *Proteobacteria*
GAM42a	GCC TTC CCA CAT CGT TT	35	γ- *Proteobacteria*
Nso190	CGA TCC CCT GCT TTT CTC C	55	Ammonium-oxidizing β- *Proteobacteria*
NEU653[b]	CCC CTC TGC TGC ACT CTA	40	Most halophilic and halotolerant *Nitrosomonas* spp.
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	50	most members of the phylum *Nitrospirae*
NIT3[b]	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.

[a]Details on oligonucleotide probes are available at probeBase (Loy et al., 2007).

[b]Used with an equimolar amount of corresponding unlabelled competitor oligonucleotide probe.

3.3.4. Biomass activity measurements

Measurements of the nitrogen compounds in the liquid phase were performed during single operational cycles (Stage III, Cycle B) fixing the DO concentration at the selected values of: 2, 4, 16, 22 and 30 mg O_2 L^{-1}, respectively. These DO concentrations were reached for values above 8 mg O_2 L^{-1} by flushing pure O_2 or for values under the cited value by flushing N_2 and proportionally reducing the air flow in order to maintain the total gas flow constant .

The AOR was estimated calculating the ammonium consumption as the difference between the theoretical value of ammonium concentration if no biological activity would take place and the value measured experimentally (Mosquera-Corral et al., 2005c). The volume variation was taken into account since the feeding process took place during the whole aeration period. The nitrite oxidizing rate (NOR) was estimated using a similar procedure but referred to the nitrate formation.

3.3.5. Calculations

3.3.5.1. Free ammonia and free nitrous acid

The concentrations of NH_3 and HNO_2 were calculated at the operational temperature from the NH_4^+, NO_2^- concentrations and the pH in the bulk liquid using Eq. 3.1 and 3.2 according to the expressions proposed by Anthonisen et al., (1976):

$$C_{NH_3} = \frac{C_{NH_4^+}}{\left(\frac{e^{\frac{6344}{T+273}}}{10^{pH}} + 1 \right)} \quad (3.1)$$

$$C_{HNO_2} = \frac{C_{NO_2^-}}{\left(10^{pH}\ e^{\frac{-2300}{T+273}} \right)} \quad (3.2)$$

3.3.5.2. Maximal oxygen and ammonium fluxes through the interface liquid-granule

The oxygen was transferred from the air to the granules firstly through the gas-liquid interface and then through the liquid-granule interface. Since the dissolved oxygen concentration in the bulk liquid is determined experimentally the oxygen flux through the surface of the granules can be calculated according to Eq. 3.3.

$$J_{O_2} = k_c \; A \left(C_L - C_s\right) \tag{3.3}$$

being J_{O2} the oxygen flux (g O_2 d^{-1}); k_c the mass transfer coefficient (m d^{-1}), A the total surface of the granules (m^2); C_L the DO concentration in the bulk liquid (g O_2 m^{-3}) and C_S the DO concentration at the surface of the granule. In order to estimate the value of k_c, an empirical relation, the Sherwood number (Sh) (Eq. 3.4), obtained for spherical particles and valid for Reynolds number lower than $3 \cdot 10^5$, was used (Hooijmans et al., 1990; Garrido et al., 1997).

$$Sh = \frac{k_c \; D_m}{D_{O_2}} = 2 + \frac{0.66}{\left(1 + \left(0.86 \, Sc^{1/6}\right)^3\right)^{1/3}} \frac{\left(Pe\right)^{1.7}}{1 + \left(Pe\right)^{1.2}} \tag{3.4}$$

being Sc and Pe the dimensionless modules of Schmidt and Peclet calculated according to Eq. 3.5 and 3.6, respectively.

$$Sc = \frac{\nu}{D_{O_2}} \tag{3.5}$$

$$Pe = Re \;\; Sc = \frac{v_g \; D_m}{D_{O_2}} \tag{3.6}$$

being v the kinematic viscosity: 10^{-6} m^2 s^{-1}, D_{O2} the oxygen diffusion coefficient in water $1.7 \cdot 10^{-9}$ m^2 s^{-1} (Picioreanu et al., 1997), v_g was estimated as the settling velocity of the granules and D_m the weighted mean diameter of the granules obtained from a sample of *n* particles (*n* ranged between 300 and 400 granules). This mean diameter was obtained by means a weighted mean volume according to Eq. 3.7.

$$\left(D_m/2\right)^3 = \frac{\sum_{i=1}^{n} \left(D_i/2\right)^3}{n} \tag{3.7}$$

being D_i the measured diameter of each granule of the sample. The specific surface of the granules a (m^2 m^{-3}) was obtained as the total surface of a sample of "n" granules divided by their total volume (Eq. 3.8).

$$a = \frac{\sum_{i=1}^{n} 4 \, \pi \left(D_i/2\right)^2}{\sum_{i=1}^{n} \frac{4}{3} \, \pi \left(D_i/2\right)^3} \tag{3.8}$$

The total surface of the granules A (m^2) was calculated by multiplying the total volume of the granules in the reactor by the specific surface (Eq. 3.9).

$$A = \frac{V_R \;\; X_R}{\rho_{granule}} \;\; a \tag{3.9}$$

being V_R the reactor volume (m^3), X_R the granules concentrations (g VSS L^{-1}) and $\rho_{granule}$ the density of the granules (g VSS $(L_{granules})^{-1}$). The maximal flux of oxygen, J_{O2}^{max} (g O_2 d^{-1}), was obtained considering the dissolved oxygen concentration at the granule surface as zero according to Eq. 3.10.

$$J_{O_2}^{max} = k_c \, A \, C_L \quad (3.10)$$

The maximal flux of ammonium towards the granules surface, J_N^{max} (g N d^{-1}), was estimated according to Eq. 3.11.

$$J_N^{max} = \frac{V_R \, (C_{NH4})_i}{HRT} \quad (3.11)$$

being $(C_{NH4})_i$ the ammonium concentration in the influent (g N L^{-1}) and HRT the hydraulic retention time (d).

3.4. Results and discussion

3.4.1. Reactor operation

Previous to this work the SBR was operated during two years in different operational conditions (data not shown, Mosquera-Corral et al., 2005a). Afterwards it was operated during 880 days in six different operational stages as indicated in Table 3.1.

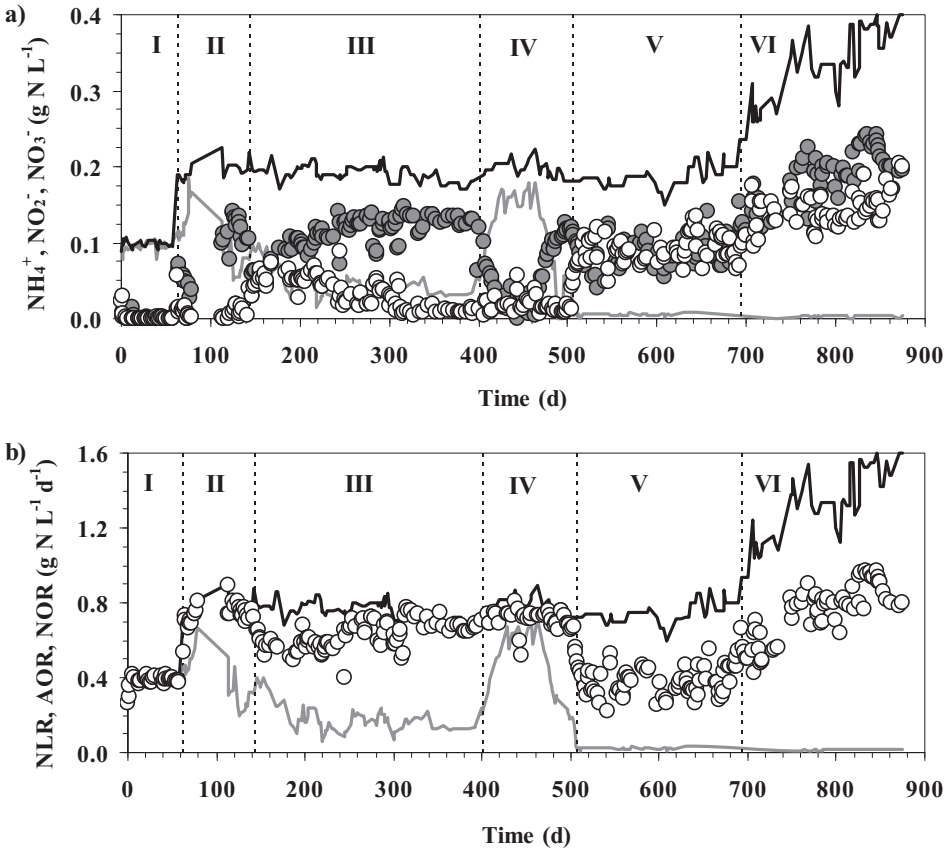


Figure 3.3. a) Nitrifying granular SBR operation in terms of NH_4^+-N concentration in the feeding (—), and NH_4^+-N (○), NO_2^--N (●) and NO_3^--N (—) concentrations in the reactor. b) Nitrogen Loading Rate (NLR) (—) Ammonium Oxidizing Rate (AOR) (○) and Nitrite Oxidizing Rate (NOR) (—) along the operational period.

During Stage I (Fig. 3.3a) the inlet ammonium concentration was of 0.1 g NH_4^+-N L^{-1} and total nitrification to nitrate was reached representing a nitrate production rate of 0.4 g NO_3^--N L^{-1} d^{-1} (Fig. 3.3b). On day 62 the inlet ammonium concentration was doubled to 0.2 g NH_4^+-N L^{-1} (Stage II). From this date on, nitrite was always present in the effluent at concentrations up to 0.1 g NO_2^--N L^{-1} meaning that the NOR was kept around 0.4 g NO_3^--N L^{-1} d^{-1} while the ammonium was almost fully depleted. During these two stages, since the feeding phase took place during 3 minutes (Cycle A), the ammonium and subsequently the estimated free ammonia concentrations were maximal during the first minutes of the cycle and concentrations as high as 10 mg NH_3-N L^{-1} were reached which have been reported as inhibitory for the nitrite oxidation process (Anthonisen et al., 1976). In order to determine the evolution of the nitrogen compounds several cycle measurements were performed corresponding to operational stages I and II (Fig. 3.4a and 3.4b). Results indicated that only when the ammonium concentration in the reactor felt to values close to zero and the free ammonia concentration reached a value close to 0.5 mg NH_3-N L^{-1} the amount of nitrite accumulated experienced a decrease. Depending on the applied nitrogen load, the concentration of free ammonia and the amount of nitrite accumulated were different.

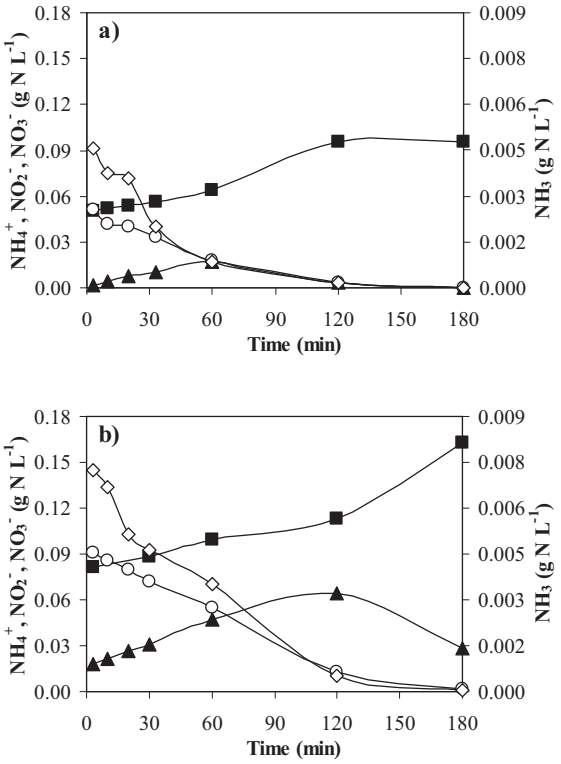


Figure 3.4. Concentrations of NH_4^+ (○), NO_2^- (▲), NO_3^- (■) and NH_3 (◇) during two cycle measurements (g N L^{-1}) a) on stage I, day 52 and b) on stage II, day 77.

To diminish the free ammonia concentrations during the first minutes of the cycle, the feeding period was extended, from day 143 on (Stage III), to 171 minutes coinciding with the aeration phase (Cycle B). The concentrations of substrates in the bulk liquid remained practically constant during the entire cycle due to the

continuous feeding pattern performed during the reaction phase. Unexpectedly, at this stage ammonium and nitrite were measured in the effluent at concentrations around 0.05 g N L^{-1} each, while nitrate concentrations remained around 0.095 g NO_3^--N L^{-1}. After day 160 the system evolved in such a way that ammonium and nitrate concentrations decreased while nitrite concentrations increased (Fig 3.3a). Between days 250 and 390, a stable operational period was achieved with average values in the effluent of 0.02 g NH_4^+-N L^{-1}, 0.13 g NO_2^--N L^{-1} and 0.04 g NO_3^--N L^{-1}. Stable nitrite accumulation was obtained although the bulk dissolved oxygen concentration was 8 mg O_2 L^{-1}.

On day 400, granules coming from another granular SBR (where complete nitrification to nitrate occurred) were added to the SBR. The amount of inoculated granules represented the 25% in dry weight of the total biomass contained in the reactor before their addition. Nitrate concentration immediately increased and after 20 days the NOR was of 0.7 g NO_3^--N L^{-1} d^{-1}. The composition of the effluent was 0.01 g NH_4^+-N L^{-1}, 0.01 g NO_2^--N L^{-1} and 0.17 g NO_3^--N L^{-1}. This total nitrification remained stable until day 460. From this day on, the nitrite concentration began to rise and 20 days later the system recovered the previous state with nitrite accumulations over 0.1 g NO_2^--N L^{-1}. These results confirmed that, in this system, full oxidation of ammonium into nitrate could not be maintained at a NLR of 0.8 g NH_4^+-N L^{-1} d^{-1} under stable conditions for long periods of time.

As it was observed that nitrite was easily accumulated in the system, even at high DO concentrations in the bulk liquid, the achievement of partial nitrification limiting the ammonium oxidation to 50% was the following objective. In order to obtain partial nitrification the DO was lowered to a medium value of 2.7 mg O_2 L^{-1} during stages V and VI. The decrease in the DO concentration avoided the activity of the NOB and lowered the activity of the AOB. Partial nitrification was achieved and an effluent with a molar NO_2/NH_4 ratio of 1.0 ± 0.3 was obtained during stages V and VI when applied NLR ranged from 0.8 to 1.6 g N L^{-1} d^{-1}.

During stage VI the ammonium concentration in the influent was stepwise increased up to 0.4 g NH_4^+-N L^{-1}. The mean temperature increased from 20 ºC to 23 ºC, coinciding with the summer station. The applied NLR was increased up to 1.6 g N L^{-1} d^{-1} and a molar NO_2/NH_4 ratio of 1.4 ± 0.3 was obtained.

3.4.2. Biomass physical properties

The biomass concentration at the beginning of the work was of 1.4 g VSS L^{-1} and it steadily increased to values around 6 g VSS L^{-1} at the end of the experiment (Fig. 3.5a). An increase of 25% of the biomass concentration was registered on period IV due to the addition of biomass from another granular SBR (indicated with the arrow in Fig. 3.5a). The percentage of ISS in the suspended solids was of 10% during the whole operational time. The concentration of solids in the effluent ranged from values as low as 0.005 g VSS L^{-1} to values up to 0.05 g VSS L^{-1}.

During the discontinuous operational stages the estimated sludge retention time (SRT) value remained below 25 d (Stages I and II) and increased up to 190 d during Stage IV, remaining over 100 d during period V and decreasing again to values around 60 d on period VI. These large SRT values were possible due to the good settling properties of the granules which guarantee the appropriated solids retention conditions to keep nitrifying bacteria inside the reactor.

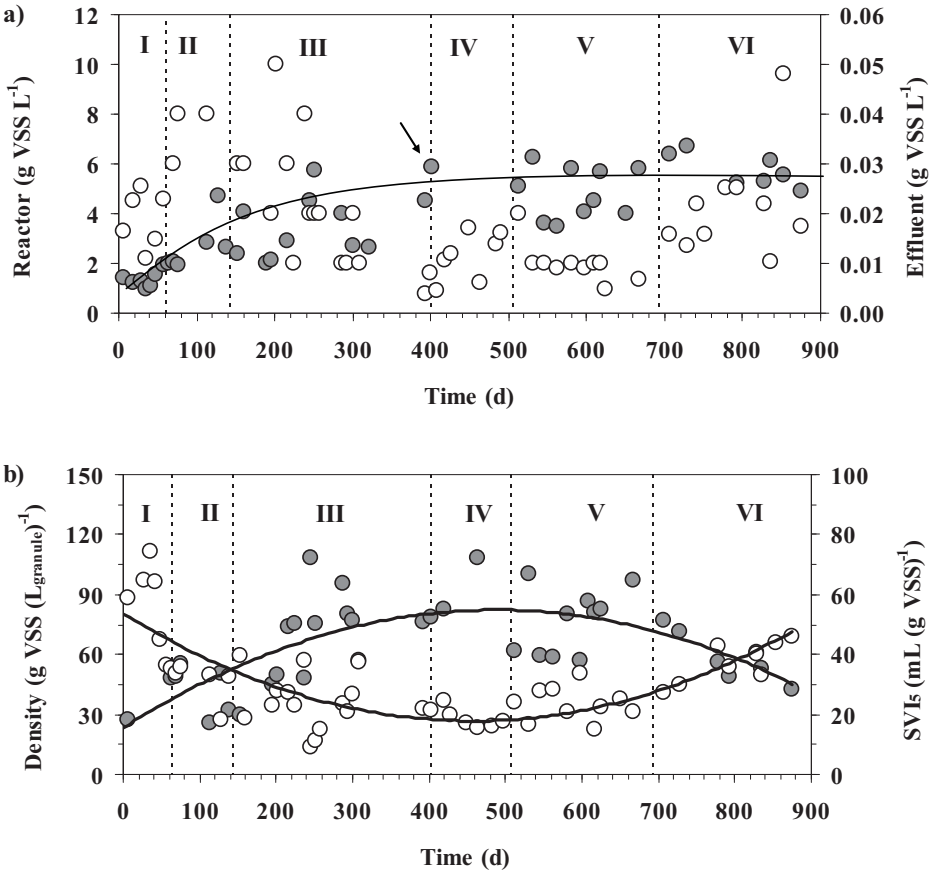
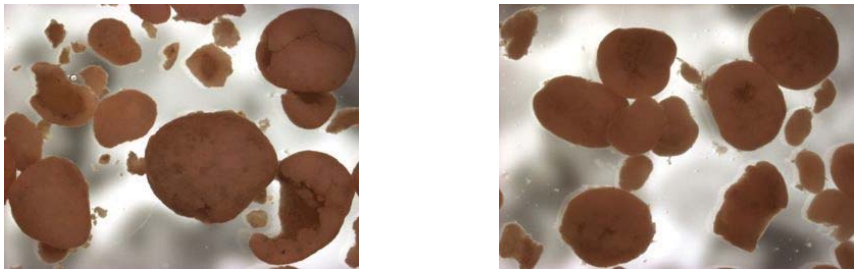


Figure 3.5. a) Concentrations of volatile suspended solids in the reactor (●) and effluent (○) (g VSS L^{-1}) of the SBR, and b) density (●) (g VSS $(L_{granules})^{-1}$) and sludge volumetric index (SVI_5) (○) (mL (g VSS)$^{-1}$) of the granules.

Regarding the settling properties of the biomass the SVI_5 and the density of the granules experienced opposite evolutions. The SVI_5 values around 100 mL (g VSS)$^{-1}$ and densities around 30 g VSS $(L_{granule})^{-1}$ were measured during stage I. The trend until stage IV was a significant improvement in the settling properties since the values of density of the granules reached values higher than 80 g VSS $(L_{granules})^{-1}$ whereas the SVI_5 decreased to values under 50 mL (g VSS)$^{-1}$ (Fig. 3.5b). These changes could be attributed to the increase in the concentration of AOB, which are known to form dense biofilms, after the two fold increase of the applied NLR. A slight decrease in the density together with a slight increase in the SVI_5 were registered during stages V and VI probably caused by the decrease in the DO concentration which affected the physical properties of the granules. Regarding the formed granules no significant differences in their sizes distributions measured as percentages of total granules surface were observed during the 880 days of operation (Fig. 3.6). The main contributors to the total surface of the granules were those with diameters between 3 and 5 mm. The mean diameter of the granules ranged during the whole operational period being between 1.9 and 3.0 mm. The

obtained granules presented large diameters which caused important limitations of oxygen due to the slight penetration of oxygen inside the granules.

a) b)



c)

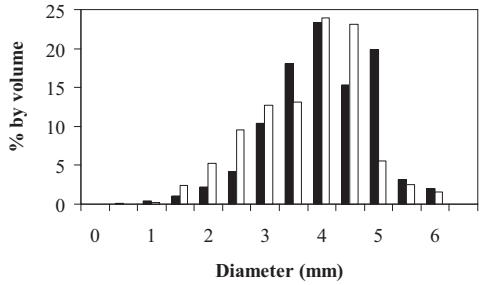


Figure 3.6. Images of the granules (zoom: 6.5x) in a) Stage I and b) Stage VI; and c) corresponding distribution of sizes in Stages I (■) and VI (□).

3.4.3. Identification of nitrifying populations

In order to gain insight in the distribution of nitrifying populations inside the granules, the FISH technique was used. Specific probes for nitrifying populations present in disaggregated and sliced granules were applied to biomass samples (Table 3.2). By application of a set of general (ALF1b, BET42a, GAM42a and EUBmix which is a mixture of EUB338, EUB338 II and EUB338 III) and specific probes (NSO190 and NEU653) to two samples of disaggregated granules collected in stages I and II, respectively, it was concluded that the genus *Nitrosomonas* was the dominant AOB present. The genera *Nitrobacter* (NIT3) and *Nitrospira* (Ntspa712) were the NOB detected, but in low concentrations in both cases.

On day 768 (stage VI) a sample of granules was collected and sliced. The NEU653, NIT3 and Ntspa712 probes were applied to these sliced granules and the results indicated that *Nitrosomonas* were located in the outermost layers of the granule and these ones were the dominant specie. *Nitrobacter* and *Nitrospira* were present but in very low amounts widespread in the outer layer of the granule (Fig. 3.7). The width of the external zone where the AOB population was present corresponded approximately to 100 μm.

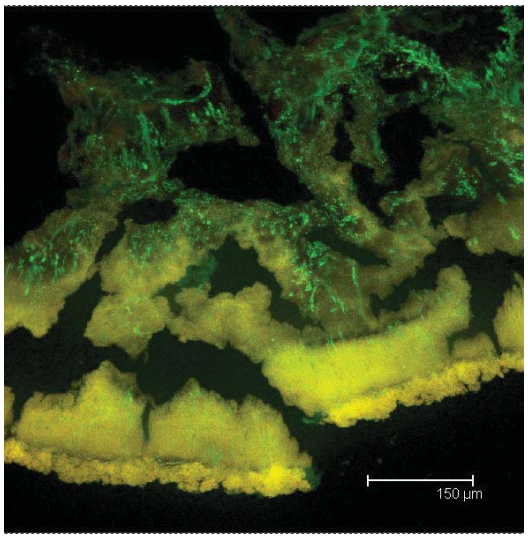


Figure 3.7. In situ hybridization of a cross-section of a granule, bright zones represent fluorescent signal of a combination of probes NEU653 and EUBmix.

3.4.4. Physical stability of the granules

During the 880 days of operation the granules kept their physical integrity and no breakage events were observed in spite of the changes in the feeding strategy. The characteristics of the granules formed were comparable to those obtained with aerobic granules grown on carbon sources. Averaging 20 different reports on aerobic granules, the mean values of SVI, density, and diameter were: 64 ± 30 mL (g VSS)$^{-1}$; 44 ± 20 g VSS ($L_{granule}$)$^{-1}$ and 1.7 ± 1.1 mm, respectively (de Kreuk et al., 2005). Therefore, the long term operation of granules treating an autotrophic medium and operating at DO concentrations in the range 2 – 4 mg O_2 L^{-1} allowed maintaining the large diameter, high density and excellent settling properties of the granules.

In the literature, nitrifying aggregates grown without carrier material have already been reported: Campos et al., (2000), accumulated 12 g VSS L^{-1} of nitrifying aggregates carrying out complete nitrification to nitrate with a SVI of 17 mL (g VSS)$^{-1}$ and a density of 100 g VSS ($L_{biomass}$)$^{-1}$; Kim and Seo, (2006) accumulated 3.5 g VSS L^{-1} of AOB with a SVI of 40 mL (g VSS)$^{-1}$ in a Sequencing Batch Airlift Reactor. However, in both cases, the mean diameter of the particles obtained was lower than 0.5 mm. By working with granules with high diameter the settling velocities of the granules is increased. Jin et al., (2008) obtained nitrifying granules with a diameter of 1.54 mm and settling velocities of 82 m h^{-1}, values significantly higher than those of activated sludge flocs (lower than 9 m h^{-1}, (Campos et al., 2000)) but lower than those of the granules from the present study which reached values up to 150 m h^{-1}.

3.4.5. Partial nitrification

The occurrence of nitrite accumulation was obtained in the granular SBR after doubling ammonium load and applying a NLR of 0.8 g N L^{-1} d^{-1}. No evolution was observed in the reactor to cope with this load increase and to produce complete nitrification. As it was previously indicated, several factors are involved in the uncoupled performance of the two steps of the nitrification, being the main ones: temperature, DO concentration and inhibition by NH_3 and HNO_2.

The nitrite accumulation registered in the system was obtained at temperatures around 20 ºC. The maximal growth rate of AOB and NOB is differently affected by the temperature due to the difference in the activation energy of both catabolic reactions. According to Wiesmann, (1994) the value of the μ_{max} corresponding to the AOB is of 0.76 and 1.97 d^{-1} at 20 ºC and 30 ºC, respectively; whereas the μ_{max} of the NOB is of 1.04 and 1.88 d^{-1} at 20 ºC and 30ºC, respectively. By working at temperatures higher than 30 ºC, the AOB have higher duplication velocities and therefore they outcompete the NOB by regulating the SRT and guarantee the occurrence of the partial nitrification. This is the operational strategy applied for example in the Sharon process (van Dongen et al., 2001). The use of SBR systems represents also an alternative to obtain partial nitrification at high temperature and it has been successfully applied, i.e., by Ganigue et al., (2007) at 36 ºC. However, partial nitrification is not only restricted to temperatures above 30 ºC and efforts have been made in order to establish partial nitrification at temperatures lower than 30 ºC. When operating at 20 ºC the AOB present maximal growth rates smaller than those of the NOB, meaning that the latter would be in advantage. In order to explain why AOB outcompete NOB in the granular SBR the possible effects of DO and the HNO_2, NH_3 inhibitions were analyzed.

3.4.6. Effects of DO

In the granular SBR the effect oxygen limiting conditions on the growth of the NOB is expected to cause the observed stable nitrite accumulation.

By working with biofilms, the importance of the biofilm surface to volume of bulk liquid ratio was highlighted as one of the critical factors in the oxygen transport from the gas phase to the granule surface. The oxygen penetration depth in biofilms varies typically in a range from 75 to 200 μm and therefore it is important to maximize the surface area of the biofilm or granule to maximize the reactor capacity (van Loosdrecht and Heijnen, 1993).

During stage III, the DO concentration in the bulk liquid was varied and monitored during several cycles in order to determine the effect of the DO on the biomass activity. By working at DO concentrations from 4 to 30 mg O_2 L^{-1} total oxidation of the ammonium fed was achieved; however, the AOB activity decreased 75% when working at DO concentration of 2 mg O_2 L^{-1} (Fig. 3.8). The NOB activity increased considerably when increasing DO concentrations up to 8 mg O_2 L^{-1}. Maximal NOB activity was not reached until attaining DO concentrations of 22 mg O_2 L^{-1}. These batch experiments clearly demonstrated the difference in the oxygen affinity constant between AOB and NOB and pointed out the role of the mass transfer limitations involved when working with granules with a so large diameter.

In the present work, the ratio between the total surface of the granules and the volume of the bulk liquid ranged between 58 and 216 m^2 m^{-3} during the reactor operation. These values are much lower than those obtained in particle based biofilm reactors of around 3000 m^2 m^{-3} (Garrido et al., 1997). It is therefore expected that the oxygen mass transport from the bulk liquid to the granule limited the oxidation rates.

In order to evaluate the maximal flux of oxygen that could be transferred from the bulk liquid to the granule, J_{O2}^{max} was calculated as the maximal flux that could be transferred through the liquid-granule interface (i.e., the flux of oxygen when the DO concentration at the surface of the granule was zero) (Eq. 3.10). The values of the mass transfer coefficient (k_c) for oxygen and the Sh module, obtained applying Eq. 3.4, 3.5 and 3.6, were $4 \cdot 10^{-5}$ m s^{-1} and 60, respectively. The maximal fluxes of oxygen and nitrogen that can be transferred to the granule were calculated from Eq. 3.7, 3.8, 3.9, 3.10 and 3.11.

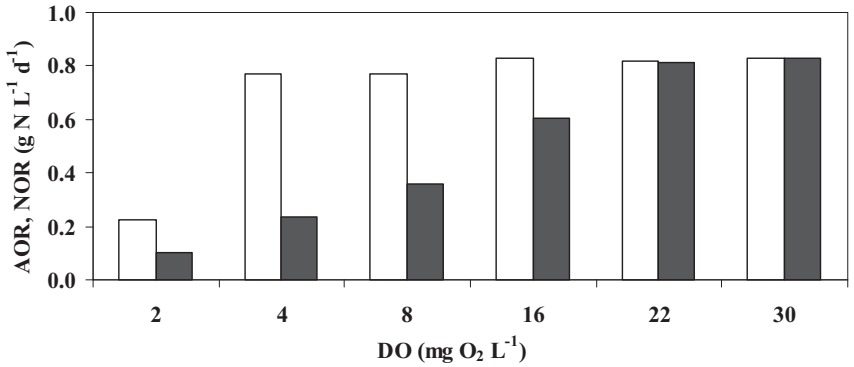


Figure 3.8. AOR (□), NOR (■) obtained at different DO in the bulk liquid of the granular SBR.

The values of the J_{O2} $(J_N)^{-1}$ ratio are presented in Fig. 3.9. The value of J_{O2} $(J_N)^{-1}$ ratio of 4.5 g O_2 (g N)$^{-1}$ corresponded to the stoichiometric value required to obtain complete nitrification which was only reached in stage I. There was a correlation between the percentage of nitrite accumulated and the value of the J_{O2} $(J_N)^{-1}$ ratio meaning that partial nitrification occurred mainly due to oxygen mass transfer limitations. Only during stages I and IV this ratio was close to the stoichiometric one necessary to obtain complete nitrification as reflected in the low percentages of nitrite accumulated. The duplication of the NLR applied caused a decrease in the J_{O2} $(J_N)^{-1}$ ratio causing nitrite accumulation. With the introduction of granules from another SBR (Stage IV) the available surface augmented with the consequent increase of the J_{O2} to values close to the stoichiometric ratio allowing significant nitrate production while almost no nitrite was present. During stages V and VI, the low oxygen flow available due to the low DO concentration in the bulk liquid allowed reaching stable partial nitrification.

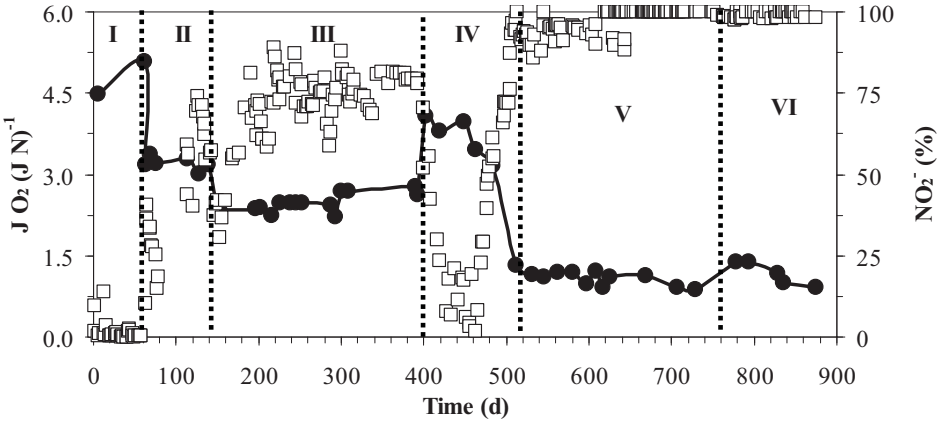


Figure 3.9. Evolution of the J_{O2} $(J_N)^{-1}$ ratio (●) and nitrite percentage in the effluent (□) along the operational period.

3.4.7. Effects of free ammonia and free nitrous acid

During stages I and II, due to the discontinuous feeding according to Cycle A, the concentrations of substrates varied along the cycle and only the maximal values are plotted in Fig. 3.10. Nevertheless, for stages III to VI the concentrations of the different substrates remained constant during the entire cycle.

During stages I to V of operation, the free nitrous acid concentrations measured remained mostly under 0.010 mg HNO_2-N L^{-1} (Fig. 3.10). No free nitrous acid inhibition of NOB was expected below this concentration (Anthonisen et al., 1976; Vadivelu et al., 2006a). Despite it was not the main selection factor, the role of HNO_2 in the partial nitrification can not be totally discarded since as it was observed by Vadivelu et al., (2006a, b) anabolism and catabolism of NOB are differently affected by the HNO_2 concentration. Values of HNO_2 concentrations as low as 0.010 mg N L^{-1} begun to reduce the growth of NOB but did not affect their catabolism. Despite the HNO_2 concentration was lower than this value during the five first periods, the mass transfer limitations occurring in the granular reactor could determine a pH profile into the granule. Important pH decreases have been reported in nitrifying aggregates caused by AOB activity (de Beer et al., 1993). The pH decrement into the granule could cause the increase of HNO_2 concentration which could affect the AOB and NOB growth. This information is not available in this work.

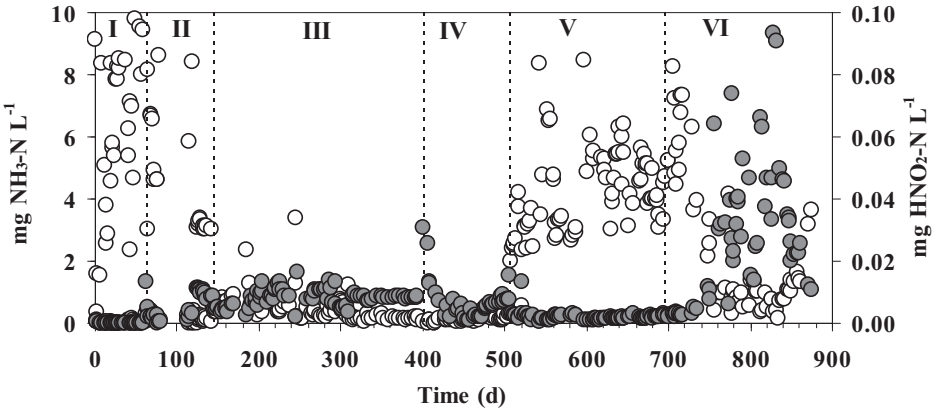


Figure 3.10. Free ammonia concentrations (○) and free nitrous acid concentration (●). For stages I and II (Cycle A) the free ammonia concentration corresponds to the value at the beginning of the cycle while the free nitrous acid corresponds to the value at the end of the cycle. For stages III to VI (Cycle B) the value is constant during the whole cycle.

Values of free ammonia of 1 mg N L^{-1} would completely inhibit NOB according to Anthonisen et al., (1976). However, several authors revealed that long term operation under high NH_3 concentration caused biomass acclimatizing increasing significantly inhibition thresholds (Turk and Mavinic, 1989). Free ammonia could affect NOB anabolism but it was not expected to be the main factor affecting the decrease in the nitrite oxidation capacity of the reactor. Its potential as main selection factor was already discarded at long term operation by several authors (Fux et al., 2004; Blackburne et al., 2008b).

3.4.8. Granular SBR as an alternative system for partial nitrification

In the present study the nitrifying granules were obtained from an aerobic granular SBR fed with an effluent with a certain COD/N. Afterwards the supplied NLR was increased until getting nitrite accumulation to finally decrease the DO concentration in the bulk liquid to limit the flux of oxygen through the liquid-granule interface.

This nitrifying granular SBR is proposed as an alternative to the Sharon process to obtain partial nitrification and produce an effluent suitable to feed an Anammox reactor at temperatures around 20 ºC. In this granular SBR, operated under important oxygen diffusion limitations and at low temperatures, similar values of the AOR as those obtained in Sharon reactors could be achieved due to the larger biomass concentrations accumulated in the reactor. Common values of biomass concentrations in a Sharon reactor are 0.1 g VSS L^{-1} (Mosquera-Corral et al., 2005b) whereas in the granular SBR much higher values around 6 g VSS L^{-1} were achieved. Moreover, the solids content in the effluent were much lower in the granular SBR of 0.02 g VSS L^{-1}. The almost absence of solids in the produced effluent avoids the possible presence of heterotrophic activity representing an advantage for the stable operation of the subsequent Anammox reactor (Jetten et al., 1998). Finally the volume required to treat a certain load will be much smaller by working with SBR technology than with the Sharon reactor as it was pointed out by Fux and Siegrist, (2004). In order to better understand the differences between both alternatives a comparison in terms of operational conditions is made below in Table 3.3.

From the analysis of the results shown in Table 3.3 it can be observed that DO concentrations required to obtain partial nitrifications are lower in the case of systems with suspended biomass than those used in biofilm systems. However, the achieved AOR by working with suspended solids reactors are in the same range than those obtained in the granular SBR.

The achievement of a partial nitrifying system can be reached by following different approaches. For example, Wyffels et al., (2004) operated a membrane bioreactor with a large SRT where the total nitrification to nitrate was performed at 30 ºC and they reduced the k_La and the HRT values to get the partial nitrification. Blackburne et al., (2008b) studied the partial nitrification with biomass in suspension using the DO as the only control parameter in a CSTR (Table 3.3). However, the system showed a marked instability in the operation registering periods when the AOB washed out and periods with the presence of NOB activity. Fux et al., (2004) also experienced difficulties in maintaining long-term partial nitrification of ammonium-rich sludge digester effluents in a moving-bed biofilm reactor at laboratory and pilot scales. These authors stated that due to the relatively long SRT presented by the biofilm systems the growth of slow-growing organisms like nitrite oxidizers can occur. When the aim is to accumulate nitrite the stable operation is difficult to maintain during long periods of time. By working at temperatures below 30 ºC, meaning that the maximal growth rate of the AOB is lower than the one for the NOB, the attempts to reach partial nitrification avoiding totally the nitrite oxidation are based on the decrease of the NOB growth rate by increasing free ammonia concentrations and/or lowering DO concentrations. These procedures usually lead, after a certain period of time, to biomass adaptation and nitrate formation.

Results obtained in the present work in terms of treated loads and concentrations of nitrogen compounds in the effluent are similar to those from previous works in systems operated with suspended biomass and with biofilms. The main difference is the stable nitrite accumulation along the time which is achieved in the nitrifying granular SBR reactor and the easy regulation of the percentage of nitrogen

compounds in the effluent by means of DO control. Furthermore the 50% composition of ammonium and nitrite is usually obtained at temperatures close to 30 ºC but in the present work this ratio is achieved at lower temperatures between 18 and 24 ºC.

Table 3.3. Comparison of operational conditions of different systems with partial nitrification.

Reactor[a]	T (ºC)	DO (mg O_2 L^{-1})	AOR (g N L^{-1} d^{-1})	AOB Activity (g N (g VSS)$^{-1}$ d^{-1})	Diameter (mm)	NH_4 / NO_2 / NO_3 (% in effluent)	Reference
MBR	30	0.1	0.5	0.05	S.S.	50 / 50 / --	Wyffels et al., (2004)
CSTR	30-40	---	1.2	---	S.S.	53 / 57 / --	van Dongen et al., (2001)
CSTR	21	1.54	0.3	---	S.S.	18 / 63 / 19	Ahn et al., (2008)
CSTR	19 – 23	0.3	0.2	---	S.S.	35 / 65 / --	Blackburne et al., (2008b)
AS	25	1.4	1.4	0.57	S.S.	5 / 75 / 20	Ciudad et al., (2005)
DHS	30	0.2	0.8	0.12	S.S.	50 / 50 / --	Chuang et al., (2007)
PBR	18-22	>3.0	1.2	---	Biofilm	-- / 100 / --	Yun and Kim, (2003)
AUFB		1.5 – 3	1.5	---	0.35	-- / 80 / 20	Tsuneda et al., (2003)
BAS	30	1.0 – 2.0	5.0	---	0.65 – 0.84	4 / 57 / 39	Garrido et al., (1997)
ITBR	22 – 26	2.0	1.1	---	0.36	3 / 60 / 37	Bernet et al., (2005)
Airlift	20 – 27	1.8-2.5	2.4	0.69	0.30 – 0.50	-- / 100 / --	Kim and Seo, (2006)
Airlift	30	>5.0	2.3	---	1.54	4 / 23 / 73	Jin et al., (2008)
SBR	18 – 24	8.0	0.7	0.26	3.00	7 / 71 / 22	This study
SBR	18 – 24	2.6 – 3.0	0.4 – 0.8	0.08 – 0.16	3.00	50 / 50 / --	This study

[a] MBR = Membrane-assisted BioReactor; CSTR = Continous Stirring Tank Reactor; AS = Activated Sludge; DHS = Down-Flow Hanging Sponge; PBR = Packed Bed Reactor; AUFB = Aerobic Upflow Fluidized Bed; BAS: Biofilm Airlift Suspension reactor; ITBR: Inverse Turbulent Bed Reactor.

3.5. Conclusions

● Stable nitrite accumulation with concentrations of 130 mg NO_2^--N L^{-1} was observed for more than 300 days in a nitrifying granular sludge reactor with dissolved oxygen concentrations around 8 mg O_2 L^{-1}, operated at room temperature (18-24 ºC) and applying a NLR of 0.8 g N L^{-1} d^{-1}.

● Carrying out experiments at different dissolved oxygen concentration, it was observed that the dissolved oxygen limited the nitrite oxidation capacity of the reactor, since by working at 30 mg O_2 L^{-1} the nitrite and ammonium oxidation capacities were similar and slightly higher than 0.8 g N L^{-1} d^{-1}.

● The inoculation of granules from another reactor allowed the complete nitrification to nitrate for a month. However, the system rapidly evolved again and recovered the previous state with nitrite accumulation demonstrating that the conditions and not the biomass history were the responsible for the low NOB activity and therefore the low nitrite oxidation capacity of the reactor. Full oxidation of ammonium into nitrate could not be maintained at a NLR of 0.8 g NH_4^+-N L^{-1} d^{-1} under stable conditions for long periods of time.

● Stable partial nitrification was obtained for more than one year producing an effluent with a NO_2^-/NH_4^+ molar ratio around 1, suitable to feed an Anammox reactor. No nitrite oxidation was registered during this period working at DO concentrations in the bulk liquid in the range 2.0—3.5 mg O_2 L^{-1} and varying the NLR from 0.8 to 1.6 g N L^{-1} d^{-1}.

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Chapter 4

Population dynamics of nitrite oxidizers in nitrifying granules[1]

Summary

The competition between *Nitrospira* and *Nitrobacter* species was analyzed in this work under conditions of excess of nitrite. A population of nitrite oxidizing bacteria (NOB) was developed from nitrifying biomass grown as granules with a mean diameter of 0.8 mm, by means of switching the composition of the feeding media from ammonium to nitrite. The initial population distribution of the granules was: 60% *Nitrosomonas* and 30% *Nitrospira* and it evolved to 45% *Nitrobacter* and 40% *Nitrospira* measured 177 days after the change in the feeding. The disappearance of *Nitrosomonas* allowed the development of an important population of *Nitrobacter* demonstrating that these microorganisms, characterized by being r-strategists NOB, are poor competitors when oxygen is the limiting substrate. Interestingly, the physical structure of the granules was not altered by the change of its microbial composition during the 220 days of operation.

[1]**Vázquez-Padín J.R., Figueroa M., Campos J.L., Mosquera-Corral A. and Méndez R**. (2009) Population dynamics of nitrite oxidizers in nitrifying granules. *Water Science and Technology* **60**(10): 2529-2536.

4.1. Introduction

Due to the slow growth rate of the nitrifying bacteria populations, nitrification is the controlling process during the biological nitrogen removal from wastewaters. Large efforts have been focused on the development of strategies to keep large nitrifying biomass concentrations inside the reaction systems by promoting the formation of biofilms or granules (Vázquez-Padín et al., 2009). In these systems both external and internal mass transfer phenomena are important and can determine the overall reaction rate (Wilen et al., 2004). Significant gradients of substrates around and inside the aggregates occur which may be responsible for the stratification of the spatial distribution and diversity of bacterial populations.

In nitrifying microbial aggregates, ammonium-oxidizing bacteria (AOB) are generally found throughout the whole aggregate while nitrite-oxidizing bacteria (NOB) are placed in the inner zones of the biofilms (Okabe et al., 1999; Vázquez-Padín et al., 2009). In this kind of systems *Nitrosomonas* are generally the dominant AOB (Schramm et al., 2000; Han et al., 2003). However, both *Nitrospira* and *Nitrobacter* are simultaneously detected but no clear reason for that has been identified (Daims et al., 2001). Traditionally, *Nitrobacter* was considered to be the most important nitrite oxidizer in WWTP. The development of microbiological tools revealed that *Nitrospira*-like bacteria are widely distributed in natural and engineered ecosystems (Burrell et al., 1998; Hovanec et al., 1998; Okabe et al., 1999; Schramm et al., 1999; Daims et al., 2001; Regan et al., 2002; Daims et al., 2006). This predominance of *Nitrospira* over *Nitrobacter* in most WWTP could be a reflection of their different survival strategies. Schramm et al., (1999) postulated that *Nitrospira*-like bacteria are K-strategists and can exploit low amounts of nitrite and oxygen much more efficiently than *Nitrobacter*. In contrast, *Nitrobacter* species are r-strategists that can grow faster than *Nitrospira*, but depend on significantly higher nitrite and oxygen concentrations. This K/r-hypothesis could explain the predominance of *Nitrospira*-like bacteria in activated sludge, where nitrite concentrations are usually low. Under these conditions the growth rate of *Nitrospira*-like bacteria is sufficiently high to maintain stable populations, while *Nitrobacter* cannot proliferate fast enough and are washed out of the continuously operated bioreactors. This fact has been already observed by Nogueira and Melo, (2006) in nitrite oxidizing chemostats and by Kim and Kim, (2006) in biofilm systems. Nevertheless other authors indicated that *Nitrospira* and not *Nitrobacter* can be the predominant populations of nitrite oxidizers in both biofilms (Okabe et al., 1999; Han et al., 2003) and granular nitrifying systems (Carvalho et al., 2006; Wang et al., 2007) even when high nitrite concentrations are present. This fact would indicate that the oxygen concentration rather than the nitrite concentration could be the key factor for the selection of nitrite oxidizing populations (Schramm et al., 2000).

4.2. Objectives

● The objective of this work was to determine the effect of oxygen availability on the distribution of nitrite oxidizing populations in a nitrifying reactor further operated as a nitrite oxidizing reactor. The performance of the system and the evolution of both physical characteristics and bacterial populations of nitrifying granules were studied.

4.3. Materials and methods

4.3.1. Reactor description

A sequencing batch reactor (SBR) with a working volume of 2.5 L, an internal diameter of 0.1 m and a height of 0.27 m was used, the height to the diameter ratio was of 2.7. The exchange volume was fixed at 20%. The hydraulic retention time (HRT) was kept at 1.25 d. Air was supplied from the bottom of the reactor through

a sparger. The duration of the operational cycles was of 6 h distributed in: aeration and feeding (345 min); settling (10 min) and withdrawal (5 min). A programmable logic controller Siemens model S7-224CPU controlled the actuations of the pumps and valves, and regulated the different phases of the operational cycles.

Temperature, pH and dissolved oxygen (DO) concentrations in the reactor were not controlled being their mean values: 21.8 ± 1.4 ºC, 8.0 ± 0.3 and 7.9 ± 0.4 mg O_2/L, respectively. The composition of the feeding medium was, in g L^{-1}: 0.084 $NaHCO_3$, 0.092 K_2HPO_4, 0.036 KH_2PO_4, 0.049 $MgSO_4$, 0.019 KCl, 0.5 mL L^{-1} of a trace solution and the nitrogen source: $ClNH_4$ (1.49 g L^{-1}) or $NaNO_2$ (0.25 - 2.71 g L^{-1}) (Table 4.1). The composition of the trace solution was in g L^{-1}: 1.50 $FeCl_3 \cdot 6\ H_2O$, 0.15 H_3BO_3, 0.15 $CoCl_2 \cdot 6\ H_2O$, 0.12 $MnCl_2 \cdot 4\ H_2O$, 0.12 $ZnSO_4 \cdot 7\ H_2O$, 0.06 $NaMoO_4 \cdot 2\ H_2O$, 0.03 $CuSO_4 \cdot 5\ H_2O$ and 0.03 KI.

Table 4.1. Main operational conditions in the different stages of the SBR reactor.

Stage	Time (days)	N-source	Inlet concentration (mg N L^{-1})
I	0-64	Ammonium	390
II	65-134	Nitrite	100
III	135-220	Nitrite	360
IV	221-285	Nitrite	500

4.3.2. Operational conditions

The reactor was previously operated for a year in order to develop nitrifying granules from an activated sludge collected from a municipal wastewater treatment plant (data not shown). At the beginning of the experiment, the nitrifying granules had a mean feret diameter of 0.8 mm and the biomass concentration was 0.7 g VSS L^{-1}. An ammonium loading rate of 0.3 g N L^{-1} d^{-1} was applied during 64 days. At day 65, the nitrogen source of the autotrophic medium was change by nitrite to suppress the activity of AOB. The nitrite loading rate applied to the systems was stepwise increased from 0.05 to 0.55 g N L^{-1} d^{-1}.

4.3.3. Analytical methods

The pH and the concentrations of DO, ammonium, volatile suspended solids (VSS) and sludge volumetric index (SVI_5) were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Nitrite and nitrate concentrations were determined by capillary electrophoresis (Vilas-Cruz et al., 1994). The morphology and size distribution, the aspect ratio and the roundness of the granules were measured regularly by using an image analysis procedure (Tijhuis et al., 1994) with a stereomicroscope (Stemi 2000-C, Zeiss) incorporating a digital camera (Coolsnap, Roper Sicientific Photometrics). For the digital image analysis the programme Image Pro Plus (Media Cybernetics) was used.

Samples of suspended biomass were collected from the reactor on operating days 0, 77, 176 and 220. The FISH technique was performed according to the procedure described by Amann et al., (1995) with 4% paraformaldehyde solution. To achieve the granular biomass breakage, biomass was sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher). The specific oligonucleotide probes used are represented in Table 4.2. The oligonucleotide probes were labelled with the fluorochromes Cy3 and FLUOS obtained from ThermoHybaid (Ulm, Germany). After in situ hybridization cells were stained with DAPI (0.5 µg mL^{-1}) for 10 min. Fluorescence signals were recorded with an acquisition system coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany). Quantification of the bacterial population was based on the procedure published by (Crocetti et al., 2002). For each hybridization experiment at least 20 randomly

chosen images were recorded, and the ratio of the area of those cells labelled by the specific probe to the area of all bacteria stained by DAPI was determined by digital image analysis using Image ProPlus. Further information about the analytical methods is provided in Chapter 2.

Table 4.2. Targeted organisms and the corresponding formamide (FA) percentages for the used oligonucleotide probes.

Probe[a]	Probe sequence (5'→3')	FA (%)	Targeted organisms
EUB338	GCT GCC TCC CGT AGG AGT	0-50	*Bacteria* domain
EUB338-II	GCA GCC ACC CGT AGG TGT	0-50	Planctomycetales
EUB338-III	GCT GCC ACC CGT AGG TGT	0-50	Verrucomicrobiales
ALF1b	CGT TCG Y(C/T)TC TGA GCC AG	20	Most α- *Proteobacteria* and other bacteria
Bet42a[b]	GCC TTC CCA CTT CGT TT	35	β- *Proteobacteria*
Pae997	TCT GGA AAG TTC TCA GCA	0	*Pseudomonas* spp.
PAR1244	GGA TTA ACC CAC TGT CAC C	20	*Paracoccus*
NEU653[b]	CCC CTC TGC TGC ACT CTA	40	Most halophilic and halotolerant *Nitrosomonas* spp.
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	50	most members of the phylum *Nitrospirae*
NIT3[b]	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.

[a]Details on oligonucleotide probes are available at probeBase (Loy et al., 2007).

[b]Used with an equimolar amount of corresponding unlabelled competitor oligonucleotide probe.

4.3.4. Calculations

The maximal oxygen amount transferred from the bulk liquid to the granule surface was calculated according to Eq. 4.1.

$$J_{O_2} = k_c \, A \left(C_L - C_S\right) \tag{4.1}$$

being J_{O2} the oxygen flux (g O_2 d^{-1}); k_c the mass transfer coefficient (m d^{-1}), A the total surface of the granules (m^2); C_L the DO concentration in the bulk liquid (g O_2 m^{-3}) and C_S the DO concentration at the surface of the granule.

The total surface of the granules A (m^2) was calculated by multiplying the total volume of the granules in the reactor by the specific surface (Eq. 4.2).

$$A = \frac{V_R \, X_R}{\rho_{granule}} \frac{6}{D_m} \tag{4.2}$$

being V_R the reactor volume (m^3), X_R the biomass concentration in the reactor (kg VSS m^{-3}), $\rho_{granule}$ the granules density (kg VSS $m_{granule}^{-3}$) and D_m the mean diameter of the granule (m). k_c was calculated following the procedure described in Chapter 3 (from Garrido et al., 1997).

The oxygen required to oxidize the nitrogen loading rate ($J_{O2}^{required}$, g O_2 d^{-1}) applied was calculated according to the stoichiometry of the ammonium oxidation (NH_4^+ + 1.5 O_2 → NO_2^- + 2 H^+, i.e., 4.57 g O_2 required per g of NH_4^+-N oxidized) and nitrite oxidation (NO_2^- + 0.5 O_2 → NO_3^-, i.e., 1.14 g O_2 required per g of NO_2^--N oxidized) processes (Eq. 4.3).

$$\left(J_{O2}^{required}\right)_I = \frac{4.57 \, C_{NH_4^+}^{inf} \, V_R}{HRT} \qquad \left(J_{O2}^{required}\right)_{II,III,IV} = \frac{1.14 \, C_{NO_2^-}^{inf} \, V_R}{HRT} \tag{4.3}$$

being HRT the hydraulic retention time (d^{-1}) and C_{NH4+}^{inf} and C_{NO2-}^{inf} the concentrations of ammonium and nitrite in influent (g N L^{-1}).

In the same way, the oxygen consumed (J_{O2}^{cons}, g O_2 d^{-1}) during the different stages was calculated according to Eq. 4.4, being C_{NO3}^{eff} and C_{NO2}^{eff} the concentrations of nitrate and nitrite in the effluent (g N L^{-1}).

$$\left(J_{O2}^{removed}\right)_{I} = \frac{\left(4.57\, C_{NO_3^-}^{eff} + 3.43\, C_{NO_2^-}^{eff}\right) V_R}{HRT} \qquad \left(J_{O2}^{removed}\right)_{II,III,IV} = \frac{1.14\, C_{NO_3^-}^{eff}\, V_R}{HRT} \tag{4.4}$$

4.4. Results and discussion

4.4.1. Biomass characteristics

The biomass concentration in the reactor remained practically constant in the range 0.5-0.7 g VSS L^{-1} during the whole operational stage in spite of changes of feeding composition. During stage I, the VSS concentrations in the effluent were around 18 mg VSS L^{-1} but those values decreased to 6 mg VSS L^{-1} when the nitrogen source was changed in feeding media (stages II-IV). The low value of VSS in the effluent allowed working at sludge retention times around 100 d from day 40 on, indicating the good retention capacity of the SBR. The obtained biomass concentration was similar to the values obtained by Kim and Kim, (2006) in nitrate oxidizing biofilm reactors (0.39 – 0.54 g VSS L^{-1}) and higher than the values obtained by Nogueira and Melo, (2006) in a chemostat (5.3 - 5.8 mg VSS L^{-1}).

The granules maintained their physical structure once nitrite was fed in the place of ammonium. No breakage was observed as demonstrated by the only slight variations registered in the granules physical properties (Fig. 1). The volume weighted mean diameter registered a slight decrease from 0.8 mm to 0.75 mm due to the disappearance of the biggest particles, larger than 2.8 mm, and the increase in importance of particles from 0.6 to 1.0 mm (Fig. 4.1). The aspect ratio and the roundness did not vary significantly remaining both values around 1.5.

The SVI_5 and the density of the granules were kept at around 20 mL (g VSS)$^{-1}$ and 77 g VSS ($L_{granule}$)$^{-1}$, respectively during the whole experiment. These values are similar to those obtained by Campos et al., (2000) who measured a SVI of 17 mL (g VSS)$^{-1}$ and a density of 100 g VSS ($L_{biomass}$)$^{-1}$ for nitrifying aggregates with 0.36 mm of diameter carrying out complete nitrification. Therefore, it can be concluded that the long term operation of nitrifying granules fed either with both ammonium or nitrite allow the maintaining of their integrity and their good settleability.

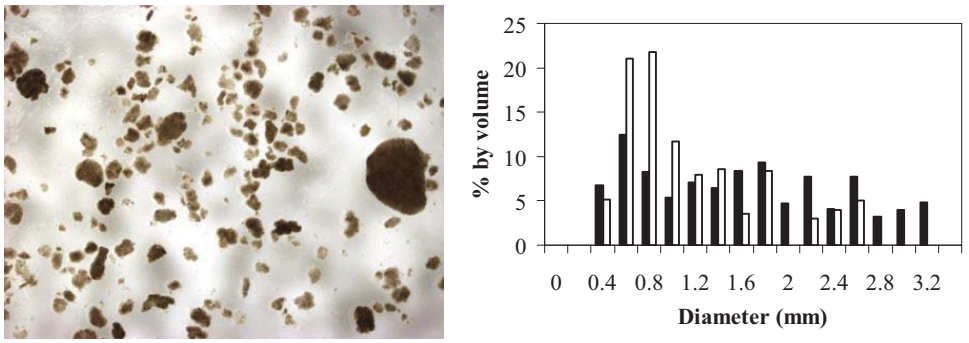

Figure 4.1. a) Image of the nitrite oxidizing granules on day 215 b) Size distribution of the granules on day 0 (■) and 270 (□).

4.4.2. Reactor performance

Ammonium was oxidized into nitrate with an efficiency of 83% during stage I. The nitrite concentration remained around 12 mg NO_2^--N L^{-1} in the effluent (Figure 4.2a). From day 65 on, a feeding media containing nitrite instead of ammonium was supplied to the system. As the NLR was increased the efficiency of nitrite oxidation decreased from 90 to 68% and, consequently, nitrite concentration in the effluent increased from 12 to 160 mg NO_2^--N L^{-1} (Stages II-IV).

Since biomass concentration, physical characteristics of granules and DO concentration in the liquid bulk did not change significantly during whole operational stage, the maximal oxygen flux through the surface of the granules was expected to keep practically constant (Figure 4.2b). However, the amount of oxygen required to oxidize all substrate applied changed due to the modifications of feeding composition. During stage I the maximal oxygen flux through the surface of the granules calculated had a similar value to that of the oxygen consumption rate which would indicate that the capacity of the system was probably limited by external oxygen transfer rate. This fact would cause that oxygen concentration inside granules were lower than during stages II-IV when the oxygen consumption rate did not exceed external oxygen transfer rate. During these stages no important DO limitation was expected to occur.

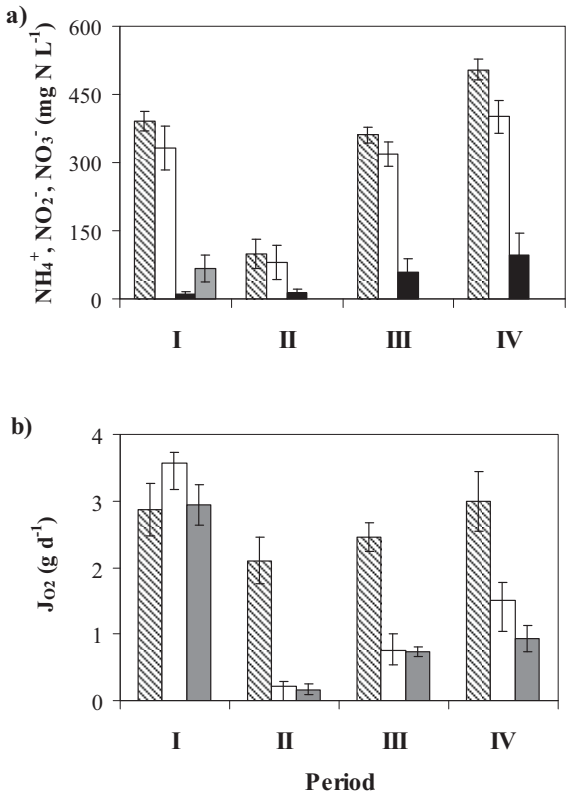

Figure 4.2. a) Concentrations of the nitrogen species in the influent (ammonium (Stage I) and nitrite (Stages II, III and IV)) (▨), and in the effluent: nitrate (□), nitrite (■) and ammonium (■). b) J_{O2}^{max} (▨), $J_{O2}^{required}$ (□) and $J_{O2}^{removed}$ (■).

4.4.3. Microbial characterization of the biomass

The evolution of the main microbial nitrifying communities in the granules was followed by FISH (Fig. 4.3).

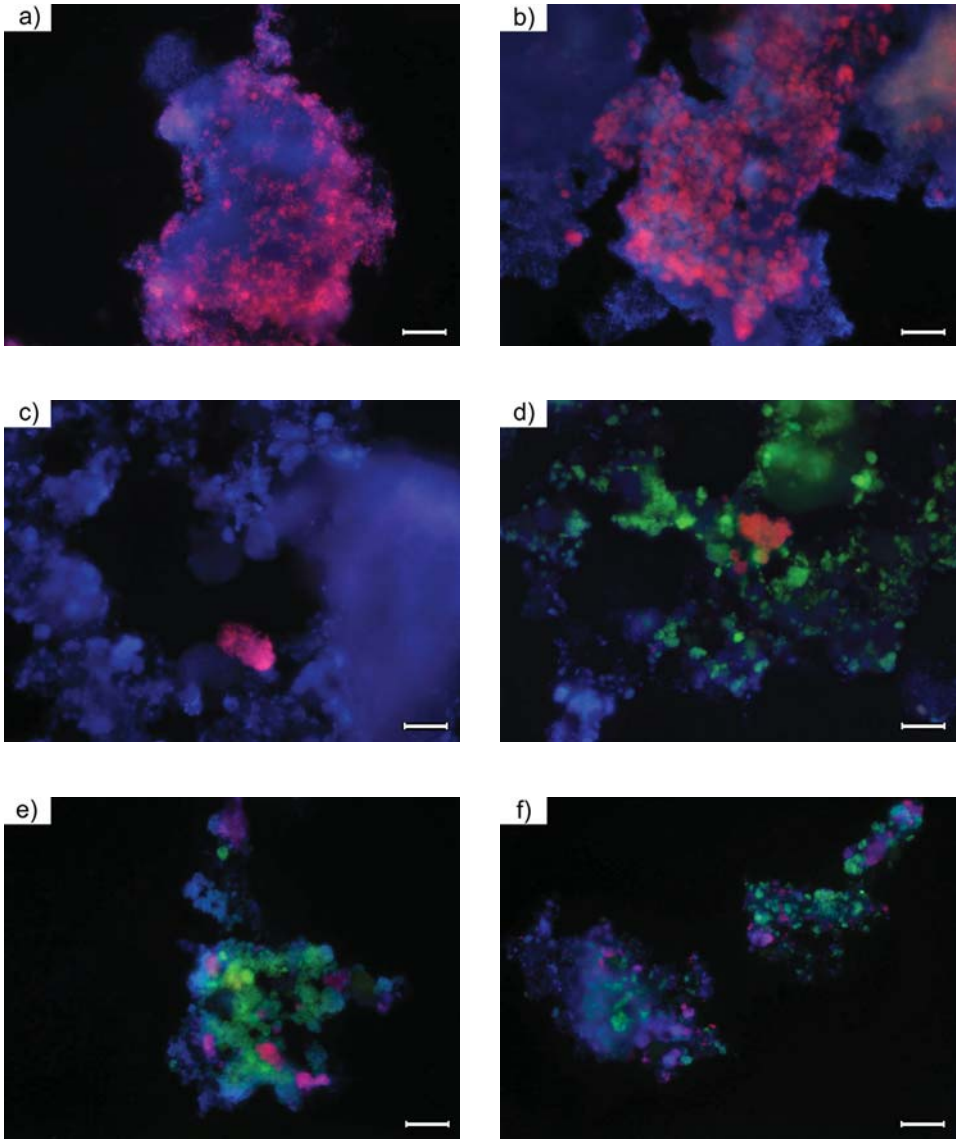

Figure 4.3. Bacterial populations on day 0: a) *Nitrosomonas* (NEU653, pink) and DAPI (blue) b) *Nitrospira* (Ntspa712, pink) and DAPI (blue). Bacterial population on day 77: c) *Nitrosomonas* (NEU653, pink) and DAPI (blue) d) *Nitrospira* (Ntspa712, green); *Nitrobacter* (NIT3, pink) and DAPI (blue). Bacterial populations e) on day 176 and f) day 220: *Nitrospira* (Ntspa712, green); *Nitrobacter* (NIT3, pink) and DAPI (blue). The bar represents 25 μm.

Samples collected on day 60 (stage I) were disaggregated and analyzed by FISH to identify the AOB and NOB populations. Analysis with the probe NEU653 showed that the granules were mainly constituted by clusters of densely packed *Nitrosomonas* that represented 60% of the bacterial population (Fig. 4.3 and 4.4). The probes Nit3 and Ntspa712 were also applied and only bacteria belonging to the phylum *Nitrospirae* were detected. This NOB represented 30% of the whole bacterial populations.

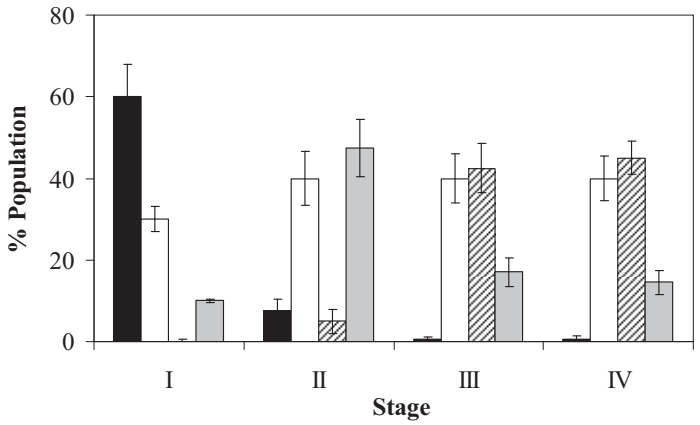

Figure 4.4. Evolution of the bacterial populations distribution of *Nitrosomonas* (NEU653, ■), Nitrospira (Ntspa712, □), Nitrobacter (NIT3, ▨) and others (the difference between the DAPI signal, which represents 100% of bacterial populations, and the sum of the positive signals detected with NEU653, Ntspa712 and NIT3, ■).

During stage II, the amount of AOB in the biomass drastically decreased. The performed FISH analysis showed that less of the 10% of the microbial population hybridized with probe NEU653. Compared to the first sample taken on day 60, the majority of the *Nitrosomonas* were no longer present in the reactor or at least not active according to the FISH results. The active 10% fraction could have survived carrying out another processes, e.g., metabolizing organic compounds (Ahn, 2006). With regards to NOB population, an increase in the *Nitropira* percentage up to 40% was observed and *Nitrobacter* were detected but only as a 5% of the total. The remaining percentage (45%) could be composed by inactive or dead *Nitrosomonas* trapped in the granules or by heterotrophs which likely grew on cell soluble microbial products (humic and fulvic acids, polysaccharides, proteins, amino acids, etc.) released during *Nitrosomonas* endogenous respiration (Barker and Stuckey, 1999). Although probes Par1244 and Pae997 for *Paracoccus* and *Pseudomonas* were applied in order to identify possible heterotrophs, no positive results were observed.

During stage III, the percentage of AOB was practically less of 1% while the *Nitrobacter* percentage in the total biomass increased to values around 45%, remaining *Nitrospira* at 40%. No significant changes of percentages of the different populations were observed on Stage IV.

The nitrite and oxygen affinity constants for *Nitrospira*-like bacteria (0.14 mg NO_2^--N L^{-1} and 0.13 mg O_2 L^{-1}) are lower than those of *Nitrobacter* (7 mg NO_2^--N L^{-1} and 1.98 mg O_2 L^{-1}) (Schramm et al., 1999; Manser et al., 2005) which cause that the former have competitive advantage at low substrates concentrations. *Nitrospira* are generally the NOB predominant in continuous biofilm systems even when nitrite is present at high concentrations, i.e., even with stable nitrite accumulation during the reactor operation (Table 4.3). In these

systems oxygen concentrations rather than nitrite concentrations could be the selective parameter for NOB. During continuous operation the substrates consumption rates are almost constant meaning that their profiles inside the biofilm remain also constant. Oxygen is generally consumed by ammonium or heterotrophic biomass located in the outer layers being scarcely present in the deeper layers of biofilm. On the other hand in SBR systems, substrates concentrations change along the time according to the operational phases which provoke concentration profiles. This fact could provoke that, in some cases, higher oxygen concentrations could be available for bacteria located in deeper layers during some stages of time. These alternating conditions could explain that there is not a clear predominant NOB type in this kind of systems but coexistence between populations.

Table 4.3. *Nitrospira* and *Nitrobacter* distribution in different systems.

System	Flow	NOB detected	Nitrite accumulation	Reference
Biofilm	Continuous	*Nitrospira*	-------	Cortes-Lorenzo et al., 2006
Biofilm	Continuous	*Nitrospira*	-------	Daims et al., 2001
Biofilm	Continuous	*Nitrospira*	Stable (up to 600 mg N L^{-1})	Han et al., 2003
Biofilm	Continuous	*Nitrospira*	Stable (up to 45 mg N L^{-1})	Okabe et al., 1999
Biofilm	SBR	*Nitrospira* + *Nitrobacter*	-------	Daims et al., 2001
Granular	SBR	*Nitrobacter*	--------	Wilen et al., 2004
Granular	SBR	*Nitrospira*	Transitory (up to 24 mg N L^{-1})	Carvalho et al., 2006
Granular	SBR	*Nitrospira*	Stable (up to 15 mg N L^{-1})	Wang et al., 2007
Granular	SBR	*Nitrobacter*	Stable (up to 120 mg N L^{-1})	Shi et al., 2009

In this work, a SBR system was operated with a long feeding/reaction stage which could be considered as a continuous system. This implies that substrates concentrations were constant along the whole operational cycle. The nitrite concentration was kept over 12 mg N L^{-1} to avoid nitrite to become the limiting substrate Nogueira and Melo, (2006). Oxygen was the limiting substrate during stage I when *Nitrospira* was the only NOB present. As soon as oxygen was not limited *Nitrobacter* appeared. These results agree with those of Schramm et al., (2000) who observed that *Nitrobacter* was the dominant NOB in the oxic part of a membrane-bound biofilm system operated under no limitation of nitrite whereas *Nitrospira* was dominant in the oxic-anoxic part.

4.5. Conclusions

● The nitrifying granules are able to maintain their structure fed with nitrite as energy source and only minor changes were registered in their physical properties.

● By switching the nitrogen source from ammonium to nitrite, a change in the biomass populations of the nitrifying granules was observed. At the beginning of the operation, *Nitrosomonas* was identified as the main population in a proportion of 60%, *Nitrospira* being the only NOB representing a 30%. After 220 days of operation with nitrite as nitrogen source *Nitrospira* and *Nitrobacter* coexisted in similar proportions around 40-45%.

● Oxygen availability inside the granules rather than nitrite concentrations seemed the main factor determining the distribution of NOB populations.

● The identification of bacterial populations and their distribution under different operational conditions allows a deeper understanding of the system performance.

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Chapter 5

Granular systems to improve Anammox biomass retention[1]

Summary

Appropriate biomass retention in reactor systems is a crucial factor for the accurate operation of the Anammox process due to the slow growth rate of this bacterial population. In the present work, the formation of granular biomass using influents with high inorganic salts concentrations by production of saline precipitates acting as promoters for biomass aggregation was studied to improve Anammox biomass retention minimizing wash-out events.

A reduction of biomass wash-out in the effluent from 50 mg VSS L^{-1} to values as low as 18 mg VSS L-1 was registered. As a consequence the biomass concentration increased significantly inside the reactor from 1 g VSS L^{-1} to 1.6 g VSS L^{-1} in 54 days. The specific Anammox activity of the biomass was not significantly affected by the presence of NaCl concentrations up to 10 g L^{-1} and varied in the range 0.35–0.45 g N (g VSS)$^{-1}$ d^{-1}. Therefore, for the improvement of the biomass retention the precipitation option is recommended for waters with high salt content.

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[1]**Fernández, I., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., and Méndez, R.** (2008) Biofilm and granular systems to improve Anammox biomass retention. *Biochemical Engineering Journal* **42**: 308-313.

5.1. Introduction

The removal of the nitrogen present both in municipal and industrial wastewaters (basically as ammonium) is carried out conventionally by means of nitrification and denitrification processes. This procedure is suitable for the treatment of wastewaters containing nitrogen and rich in biodegradable carbon, but it results expensive for the treatment of wastewaters with low carbon to nitrogen ratios, like rejection waters from anaerobic sludge digestion. The treatment of these effluents involves important amounts of dissolved oxygen for nitrification and addition of an external source of organic matter for the denitrification step with the consequent increase of operational costs. The use of a combined system composed by a process of partial nitrification of ammonium to nitrite and the Anammox process leads to a reduction of costs. The Anammox (ANaerobic AMMonium Oxidation) is an autotrophic process which consists in a combination of ammonium and nitrite by Planctomycete-type bacteria under anoxic conditions to generate nitrogen gas (Strous et al., 1999).

The application of the Anammox process is limited by its long start-up stages due to very low growth rates and biomass yields of the involved biomass (Jetten et al., 1997). Minimizing the wash-out of the biomass in the effluent by improving its retention becomes critical when biomass with so long duplication time around 11 d (Strous et al., 1998) is used. Systems where the improvement of biomass retention is achieved reduce the duration of the start up stage and provide better conditions to implant the Anammox process at industrial scale. In reactors where biomass grows in form of biofilms or granules, the formation of compact aggregates increases the settling velocity of the biomass and improves its retention and the amount of biomass growing in suspension is minimized (Arrojo et al., 2004).

The aggregation of biomass can be promoted also by means of favouring the precipitation of inorganic salts. According to the DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory when the two surfaces carry a charge of the same sign, there is a free energy barrier between them which acts as a repulsive force (Maximova and Dahl, 2006). This force seriously prevents the approach of one cell to another. The increase of the ionic strength of the medium would decrease the electrical repulsion, by compressing the double layer, and could initiate cell-to-cell interaction. Therefore, an increase of the salinity can favour the granulation. Moreover, the solubility product is also influenced by the salinity of the medium and its increase would enhance the precipitation of the salts less soluble which could act as nuclei for granulation.

5.2. Objectives

- In the present work the effects of the presence of high inorganic salt concentrations in feeding medium to provoke precipitation were tested to improve the retention of Anammox biomass in a sequencing batch reactor.

- The effects of NaCl concentration up to 10 g L^{-1} on the Anammox activity and on the physical properties of the formed aggregates was also tested.

5.3. Materials and methods

5.3.1. Reactor description

A sequencing batch reactor (SBR) with a working volume of 2 L, an internal diameter of 0.1 m and a height of 0.27 m was used, the height to the diameter ratio being 2.7 (Fig. 5.1). Temperature was controlled at 33 ºC by using a thermostatic jacket and pH was not controlled and ranged between 7.5 and 8.0. The complete mixture inside the reactor was achieved using a mechanical stirrer with rotating speed of 100 rpm. To avoid the presence of oxygen in the reactor; a low flux of argon (95% Ar and 5% CO_2) was bubbled in the system. A programmable logic controller Siemens model S7-224CPU controlled the actuations of the pumps and valves, and regulated the different phases of the operational cycles (Fig 5.2).

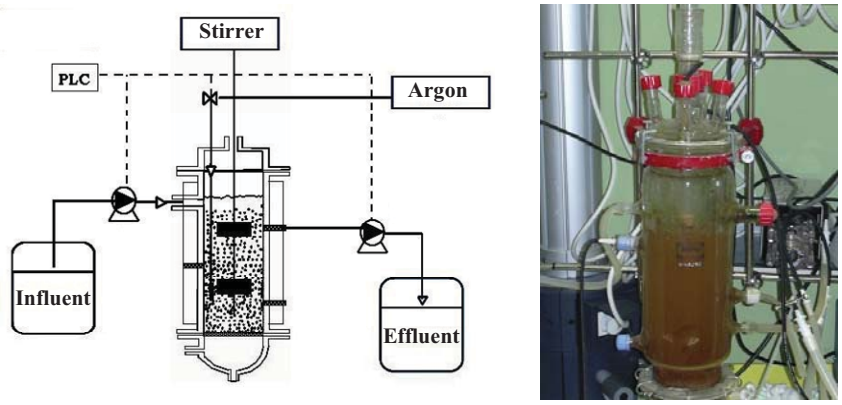

Figure 5.1. Scheme and photograph of the SBR.

The reactor was operated in cycles of 6 h (Fig. 5.2) distributed in four stages: mixed fill (300 min), mix (30 min), settle (15 min) and draw (15 min) according to Dapena-Mora et al., (2004a). The exchange volume was fixed at 12.5%. The Hydraulic Retention Time (HRT) was maintained at 2 d. The settleable solids present in the effluent were returned to the reactor.

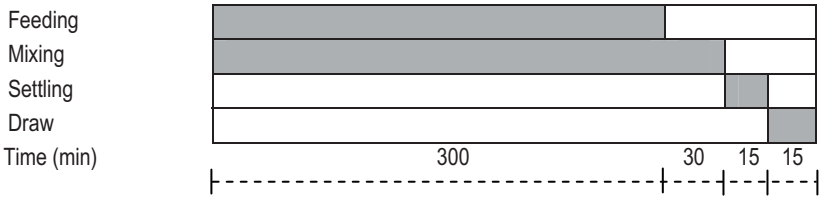

Figure 5.2. Distribution of the operational cycle.

5.3.2. Composition of the feeding media

The SBR was fed with a synthetic autotrophic medium using $NaNO_2$ and $(NH_4)_2SO_4$ as nitrite and ammonium sources in three different operational stages. The Nitrogen Loading Rate (NLR) applied was kept constant at 0.4 g N L^{-1} d^{-1} and the salinity of the feeding media was increased during the operation (Table 5.1).

Table 5.1. Characteristics of the feeding media used in the different operational stages.

Stages	Days	NaCl (g L^{-1})	NH_4^+-N (mg L^{-1})	NO_2^--N (mg L^{-1})	NLR (g N L^{-1} d^{-1})
I	0-18	0	400	400	0.4
II	18-50	5	400	400	0.4
III	50-80	10	400	400	0.4

The composition of the synthetic autotrophic medium was as follows in g L^{-1}: 1.25 $KHCO_3$, 0.005 NaH_2PO_4, 0.30 $CaCl_2 \cdot 2H_2O$, 0.20 $MgSO_4 \cdot 7H_2O$, 0.00625 $FeSO_4$, 0.00625 EDTA, 0.15 mL L^{-1} H_2SO_4 and 1.25 mL L^{-1} of a trace solution. The composition of the trace solution was in g L^{-1}: 15 EDTA, 0.43 $ZnSO_4 \cdot 7$ H_2O, 0.24 $CoCl_2 \cdot 6$ H_2O, 0.99 $MnCl_2 \cdot 4$ H_2O, 0.25 $CuSO_4 \cdot 5$ H_2O, 0.22 $NaMoO_4 \cdot 2$ H_2O, 0.19 $NiCl_2 \cdot 6$ H_2O, 0.21 $NaSeO_4 \cdot 10$ H_2O, 0.014 H_3BO_3 and 0.05 $NaWO_4 \cdot 2$ H_2O.

5.3.3. Inocula

The reactor was inoculated with enriched Anammox sludge from a laboratory scale SBR operated at the University of Santiago of Compostela (Dapena-Mora et al., 2004b). The initial concentration of biomass was 1.0 g VSS L^{-1}. The initial specific Anammox activity of the biomass was 0.4 g N (g VSS)$^{-1}$ d^{-1}.

5.3.4. Analytical methods

The pH, ammonia, volatile suspended solids (VSS), inorganic suspended solids (ISS) and sludge volumetric index (SVI_5) were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Nitrite and nitrate concentrations were determined by capillary electrophoresis (Vilas-Cruz et al., 1994). DO concentration was measured with a dissolved oxygen probe (AQUALITYC, model OXI-921) connected to a meter (M-Design Instruments TM-3659).

The distribution of particle size was measured using an Image Analysis procedure (Tijhuis et al., 1994). The morphology and size distribution of the granules were measured regularly by using an image analysis procedure with a stereomicroscope (Stemi 2000-C, Zeiss) provided with a digital camera (Coolsnap, Roper Sicientific Photometrics). For the digital image analysis the programme Image Pro Plus was used. The elemental analysis of the surface of the biomass aggregates was made using a transmission electron microscope (TEM) (PHILIPS CM12). Further information about the analytical methods is provided in Chapter 2.

5.3.5. Specific Anammox activity tests and specific nitrogen loading rate

Batch experiments to determine the specific Anammox activity (SAA) were performed according to the methodology described by Dapena-Mora et al., (2007), based on the measurement along time of the overpressure generated in closed vials by the nitrogen gas produced as detailed in Chapter 2. For stages II and III where NaCl was present in the influent, two different tests were performed, one using a medium without NaCl and the other one using the medium composition in terms of NaCl concentration corresponding to each stage.

The Specific Nitrogen Loading Rate (SNLR) was calculated dividing the Nitrogen Loading Rate (NLR) by the concentration of VSS in the reactor at the moment of the calculation.

5.4. Results and discussion

5.4.1. Reactor operation

The experiments in the SBR were performed during 72 days. The nitrite (which is the limiting substrate since the feeding media contained ammonium and nitrite in a ratio 1:1) was almost fully depleted along the cycle and the concentration of ammonium in the effluent was practically constant at a value of 100 mg NH_4^+-N L^{-1} (Fig. 5.3). The NLR applied during the whole operation of the SBR was around 0.4 g N L^{-1} d^{-1}. No significant effect was observed in the effluent composition due to the presence of the different NaCl concentrations (5 and 10 g L^{-1}) tested in the feeding media.

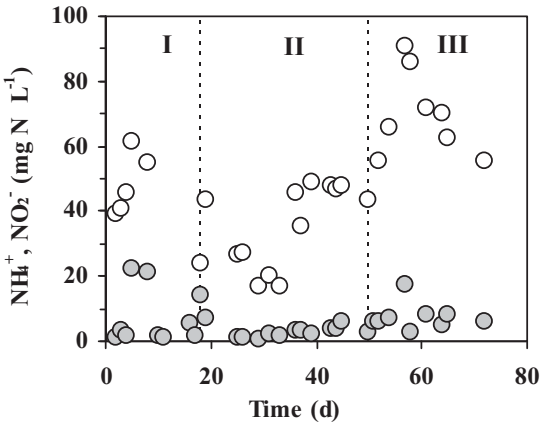

Figure 5.3. Ammonium (○) and nitrite (●) concentrations in the liquid phase of the SBR.

In order to prove that the system was not overloaded the SNLR applied to the reactor and the SAA of the biomass obtained from batch tests were compared following the same procedure as Dapena-Mora et al., (2004c). The SNLR applied to the SBR during its operation was always below the maximum specific activity of the biomass which ranged between 0.35 and 0.45 g N (g VSS)$^{-1}$ d^{-1} (Fig. 5.4). This fact indicates that the system was operated close to be overloaded only during stage I. During the rest of the operational stages not only no biomass wash-out was observed but also an important increase of the biomass concentration was measured which explains the decrease in the SNLR while the NLR was kept constant.

The SAA of the biomass decreased from 0.4 to 0.35 g N (g VSS)$^{-1}$ d^{-1} when 5 g L^{-1} of NaCl were added but when the concentration of salt was increased up to 10 g L^{-1} the SAA increased to 0.45 g N (g VSS)$^{-1}$ d^{-1} which is slightly higher than the initial value (Fig. 5.4). These changes of the SAA values are not correlated to the salinity of the medium and could be due to the necessity of an adaptation period. This adaptation process is needed when adding salt to biomass non-adapted but also when removing salt of salt-adapted biomass. The batch activity assays to determine the SAA showed no difference with or without salt addition in the medium during stage II (5 g NaCl L^{-1}). However, during stage III the batch assays performed without salt addition in the medium showed a decrease in the SAA of 20% corroborating the need of an adaptation period.

The adaptation of Anammox biomass to high salt concentrations observed in the present work agrees with the results obtained in previous works. Kartal et al., (2006) observed that adapted Anammox biomass

maintain some activity at salt concentrations up to 30 g L^{-1}. Dapena-Mora et al., (2007) performed batch tests to study the effect of different salts on the specific activity of Anammox biomass non-adapted to high concentrations of salts. They observed that 3 mg L^{-1} of NaCl enhanced the Anammox activity; an increase in the SAA of 20% was obtained. However, a NaCl concentration of 10 mg L^{-1} decreased slightly the Anammox activity to 90% of its initial value in absence of salt.

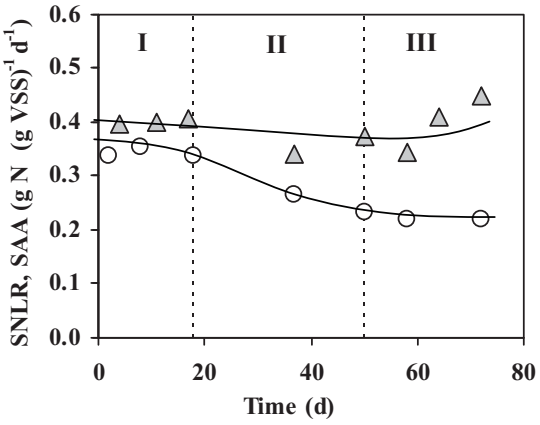

Figure 5.4. Specific Nitrogen Loading Rate (SNLR) applied (○) and Specific Anammox Activity of the biomass (SAA) (▲).

The values of SAA obtained in the present work were similar to those found by Dapena-Mora et al., (2004c) operating a SBR with granular biomass. In the same work, Dapena-Mora et al. (2004c) used a gas-lift reactor and observed values as high as 0.9 g N L^{-1} d^{-1}, however the stability of their system was not very good and both, overloads and biomass flotation events took place.

5.4.2. Biomass retention

The biomass concentration was constant in the SBR (Fig. 5.5) during stage I which was an indication of the existing balance between production and wash-out of biomass.

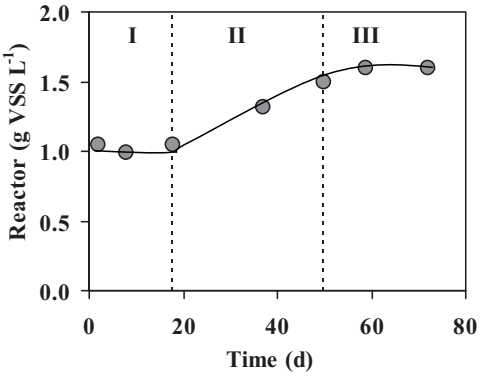

Figure 5.5. Concentration of biomass in the reactor (g VSS L^{-1}) (●).

Once the NaCl was added in the feeding (stages II and III) the biomass concentration increased steadily in 0.6 g VSS L^{-1} within 54 days. The productivity of Anammox sludge obtained from the stoichiometry of the process given by Strous et al. (1998) was 0.038 g VSS produced per g NH_4^+-N consumed. Therefore, 0.82 g VSS would have been produced during this interval of operation of the reactor. Assuming that the decay of the biomass is neglected the amount of biomass retained in the reactor was the 73 % of the stoichiometrically produced amount.

After NaCl addition, the concentration of solids in the effluent decreased significantly and, as a consequence, SRT values of 50 days were obtained (Fig. 5.6).

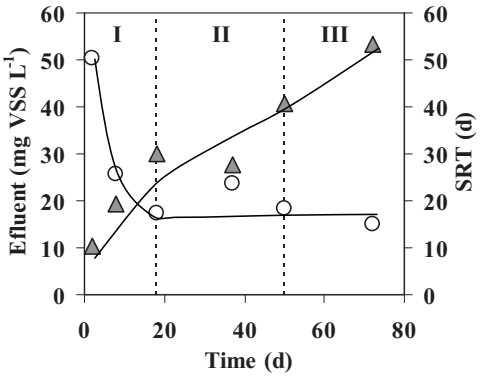

Figure 5.6. Biomass concentration in the effluent (○) and sludge retention time (SRT) (▲).

5.4.3. Reasons for the improvement of biomass retention

In order to establish the different phenomena involved in the improvement of the biomass in the reactor, the characteristics of the obtained biomass aggregates were analysed.

The percentage of inorganic fraction in the biomass, as the ratio between ISS and TSS, increased from the 10% in the stage I until 21% in stage III. This inorganic fraction is attributed to the precipitation of inorganic salts due to the NaCl addition. The precipitation was achieved increasing the salinity of the media and therefore varying the equilibrium and the solubility of the products. Elemental analysis of the precipitates present in the surface of the granules indicated that they were mainly composed by Ca_3PO_4 which is characterized by its very low solubility. These precipitates could easily act as precursors for the formation of Anammox granules, being a support material where the biomass could attach, or this precipitation could also occur directly on the surface of already formed aggregates. Thus the improvement of the settleability of the granules can be directly related to this inorganic precipitation which caused an increase of the density of the aggregates producing sludge with good SVI_5 values: 52 mL $(g\ VSS)^{-1}$ at the end of stage III (Fig. 5.7).

Although the precipitates play an important role as support material, in the conditions described it can also represent a problem if it occurs in a large extension on the surface of already formed aggregates. In such a way, if the amount of formed precipitates is too high the whole surface of the aggregates can be covered and the activity of the biomass reduced due to the increase of diffusional resistance (Trigo et al., 2006).

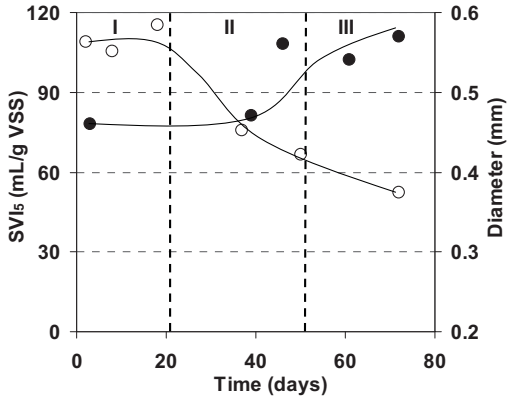

Figure 5.7. Sludge volumetric index (○) and average feret diameter (●).

Concerning the biomass physical characteristics, the feret diameter increased from 0.47 to 0.58 mm (24% of increase) and the SVI_5 decreased from 120 to 50 mL (g VSS)$^{-1}$ indicating the compacting of the biomass (Fig. 5.7). Therefore, the capacity of the system to retain biomass increased.

Visual observation of the biomass samples indicated that during stage I the biomass forming small flocs and in suspension was predominant (Fig. 5.8). During stages II and III no biomass in suspension was observed and the small flocs became aggregates. This behaviour was already observed by Campos et al., (2002) working with nitrifying sludge.

5.4.4. Applications

The external addition of salt to enhance granulation of Anammox biomass might not be feasible at industrial scale due the cost of reagents. But the use of wastewater containing high salt concentrations is a possibility. In the industry effluents with high salt content are relatively common, as those produced in the fish canning industry, due to the use of sea water during the manufacturing processes. These effluents are firstly treated in an anaerobic digestion stage and then a treatment for nitrogen removal is necessary. Therefore, the application of the Anammox process for their treatment could be advisable together with the use of the salt content to promote precipitates and to produce good settling aggregates. Although the promotion of precipitates must be carefully controlled to avoid the precipitation of extremely high amounts of salts, which could diminish the activity of the Anammox biomass as it was previously observed in the case of anaerobic sludge (Van Langerak et al., 1998).

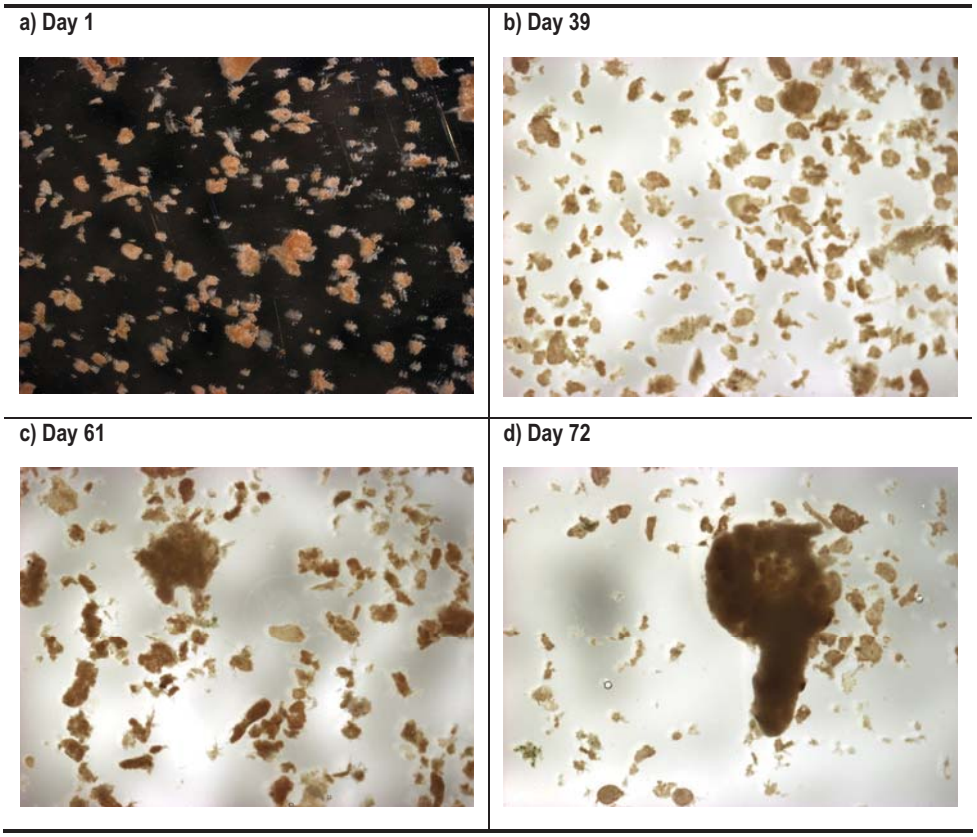

Figure 5.7. Stereomicroscope images (zoom: 10x) of biomass in the SBR in different operational days.

5.5. Conclusions

● Improvement of the Anammox biomass retention, indicated by an increase of biomass concentration inside SBR via dissolved salt addition up to 10 g NaCl L^{-1} for precipitate production.

● The increase of biomass retention was correlated to an improvement of biomass settling properties (lower SVI_5 values of 50 mL (g VSS)$^{-1}$ and bigger average diameters of 0.58 mm) in SBR1. Reduction of biomass growth in suspension was observed.

● The SAA of the biomass experienced slight variations in the presence of NaCl concentrations and was maintained around 0.40—0.45 g N (g VSS)$^{-1}$ d^{-1}.

● The choice of the application of precipitation to improve Anammox biomass retention depends upon the characteristics of the water to be treated.

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Chapter 6

Development of the CANON process in a continuously aerated granular SBR and an air pulsing SBR to treat anaerobic digester supernatants at room temperature[1,2,3]

Summary

The CANON (Completely Autotrophic Nitrogen removal Over Nitrite) process was successfully developed in two sequencing batch reactors (SBR): a granular and an air pulsing reactor (SBR and SBRP) fed with the supernatant from an anaerobic sludge digester and operated at moderately low temperatures (18-24 ºC). Both units were started up as nitrifying reactors where the dissolved oxygen concentration was decreased until partial nitrification was achieved.

In the granular SBR, while partial nitrification occurred, nitrogen losses due to the spontaneous growth of Anammox bacteria inside the reactor occurred. Once the stable CANON process was established, a mean nitrogen removal rate of 0.8 ± 0.1 g N L^{-1} d^{-1} was registered. The settling velocities of the granules ranged from 70 to 150 m h^{-1} with sludge volumetric index (SVI_5) values lower than 50 mL (g VSS)$^{-1}$ during the whole operation.

The SBRP was inoculated with sludge containing Anammox biomass once stable partial nitrification was achieved. The maximal nitrogen removal rate obtained was of 0.45 g N L^{-1} d^{-1}. By working at a dissolved oxygen concentration of 0.5 mg O_2 L^{-1} in the bulk liquid, nitrogen removal percentages up to 85% were achieved. The biomass content in the reactor was of 4.5 g VSS L^{-1}. The ammonium oxidizing bacteria (AOB) were in the form of flocs and granules (average diameter of 1.6 mm). These granules contained Anammox bacteria (present in the inner core of the granules) surrounded by AOB (present in the external layers of the granules).

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6.1. Introduction

Nitrogen removal has gained in attention over the last decades due to the problem that its presence originates in aquatic systems, such as oxygen depletion, fish toxicity and media eutrophication. The conventional process is based on a sequence of aerobic and anoxic conditions where the ammonium is firstly oxidized to nitrate and then reduced using an organic carbon source as electron donor during the denitrification process. Efficient and sustainable processes like anaerobic digestion in many cases produce effluents highly contaminated in ammonium and poorly concentrated in biodegradable chemical oxygen demand (COD). In those cases, to carry out the conventional nitrification-denitrification processes the addition of an external carbon source is needed to reach a complete nitrogen removal.

When this is the case an alternative strategy consists of making a shortcut in the nitrogen cycle, the "nitrite route" avoiding the nitrite oxidation to nitrate and its further denitrification. This route can be carried out in two ways. One alternative consists of the partial nitrification of all the ammonium to nitrite and the subsequent denitrification with an organic carbon source. This alternative involves the saving of 25% of aeration costs, and 40% of addition of external carbon source. Furthermore lower sludge production is registered (Van Loosdrecht and Jetten, 1998).

The application of the second option, the combination of a partial nitrification followed by the Anammox process, is recommended when the treated wastewater has no biodegradable carbon source. The Anammox process consists of the anaerobic oxidation of ammonium (Van de Graaf et al., 1995; Van de Graaf et al., 1996) using nitrite as electron acceptor according to the stoichiometry described by Strous et al., (1999) (Eq. 6.1).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \quad (6.1)$$

In general it is not common to find effluents with the required composition to be treated via the Anammox process. For this reason several alternatives have been studied to obtain an effluent containing both ammonium and nitrite. To reach this objective, half of the ammonium fed to the system has to be converted into nitrite by the Ammonium oxidizing bacteria (AOB) and therefore, the oxidation of nitrite to nitrate carried out by Nitrite Oxidizing Bacteria (NOB) has to be avoided. The AOB and NOB are two phylogenetically unrelated groups whose different growth rates and the way this growth rates are affected by parameters like temperature, pH, dissolved oxygen (DO), etc. can be used to outcompete NOB and to uncouple both reaction rates.

Nitrite accumulations have been reported in several systems, for biomass growing in suspension (Blackburne et al., 2008), in biofilm (Garrido et al., 1997) or in aggregates/granules (Kim and Seo, 2006). Several strategies have been used to reach partial nitrification (Ahn, 2006):

1) Increasing free ammonia concentration working at high pH values and limiting the growth of NOB due to their higher sensitivity to free ammonia inhibition than AOB (Anthonisen et al., 1976).

2) Decreasing the dissolved oxygen concentration due to the lower oxygen affinity of the NOB compared to AOB (Wiesmann, 1994).

3) Operating at temperatures above 25 ºC since the maximum specific growth rate of the AOB will be higher than that of NOB at these conditions. In fact this is the basis of the SHARON technology which consists

of a Continuous Stirring Tank Reactor (CSTR) operated a hydraulic retention time (HRT) of around 1 day and 30 ºC to favor the growth of AOB and the washout of the NOB (Hellinga et al., 1998).

In order to apply the partial nitrification and the Anammox processes it is important to take into account that they can be performed in two different units (Sharon-Anammox processes) or in a single one, called by different names, CANON: Completely Autotrophic Nitrogen removal Over Nitrite process (Third et al., 2001), OLAND: Oxygen-Limited Autotrophic Nitrification-Denitrification (Kuai and Verstraete, 1998; Pynaert et al., 2004) or aerobic/anoxic deammonification (Hippen et al., 1997; Helmer et al., 2001). Under oxygen-limited conditions a co-culture of aerobic and anaerobic ammonium-oxidizing bacteria can be established in a single unit in a CANON system. In those systems, NOB compete for oxygen with the aerobic AOB and for the nitrite with Anammox bacteria, and thus its growth (and subsequent nitrate production) is prevented. Another reason to maintain low oxygen concentrations is that Anammox bacteria are reversibly inhibited by dissolved oxygen concentration higher than 0.5% of air saturation (Strous et al., 1997).

Several strategies have been tested to optimize the start-up of reactors where the CANON process occurred. In most of the cases the CANON process was developed from an Anammox reactor where nitrifying biomass was inoculated and low oxygen concentrations were maintained inside the system (Sliekers et al., 2003; Third et al., 2005). Another possible strategy was to start-up the reactor as a partial nitrification system under oxygen limited conditions and then to inoculate Anammox biomass as it was carried out by Pynaert et al., (2004) and Gong et al., (2007) in a rotating disk contactor or a membrane aerated reactor, respectively.

The main disadvantage of these processes relies on the low growth rate of AOB and Anammox bacteria with doubling times of 1 day (Wiesmann, 1994) and 11 days (Strous et al., 1998), respectively. To enhance the performance of reactors involving slow growing bacteria, high sludge retention times are mandatory and therefore the attachment of bacteria on a carrier material to develop biofilms or the self-aggregation concept in granules are recommended. Aerobic granulation presents the advantage of excellent settleability of the granules, high biomass retention and high resistance to toxic compounds.

The applicability of the Anammox process at full-scale has already been demonstrated. Full-scale SHARON-Anammox plants are operating in the Netherlands and Japan treating wastewaters coming from: municipal reject water, tanneries, potato processing and semi conductor industry (Abma et al., 2007; van der Star et al., 2008; Lamsam et al., 2008). The one single reactor configuration has also been implemented at full scale in Austria, Germany and Switzerland to treat anaerobic digester supernatants and demonstrated being economically viable (Wett, 2006).

6.2. Objectives

● The aim of this work relies on the development of the CANON process in a continuously aerated granular sequencing batch reactor (SBR) and an air pulsing sequencing batch reactor (SBRP) operated both at room temperature.

● The suitability of both reactors will be analyzed according to the start-up duration to achieve significant CANON capacity, the maximal nitrogen removal capacity reached and the stability of the process operation.

6.3. Materials and methods

6.3.1. Reactors description

Two sequencing batch reactors (SBR and SBRP) with a working volume of 1.5 L were used. Dimensions of the units were: height of 465 mm and inner diameter of 85 mm, the height to the diameter ratio being 5.5. The exchange volume was fixed at 50%. A set of two peristaltic pumps was used to introduce the feeding solution (on top of the reactor) and to discharge the effluent (at medium height in the column reactor), respectively. A programmable logic controller Siemens model S7-224CPU controlled the actuations of the pumps and valves, and regulated the different periods of the operational cycle.

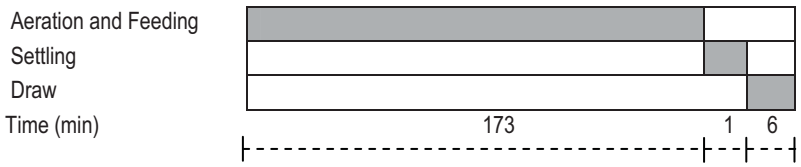
In the case of the air pulsing reactor (SBRP) air was supplied by an air pulsing device operated at a constant frequency of 0.09 s^{-1}, meaning that air is supplied in repeated cycles of one second of flow and 10 seconds without flow. The function of the air pulses consisted of providing a good mixture to the liquid media and supplying the required DO concentration for the activity of the biomass.

6.3.2. Operational conditions

6.3.2.1. Granular SBR

The operation of the Granular SBR in this chapter is a continuation of the operation described in Chapter 3 when the reactor was performing partial nitrification fed with a synthetic influent. The SBR was operated with temperature controlled at 20 ± 1 ºC by means of a thermostated bath. Air was supplied through the bottom of the reactor using an air sparger to promote the transfer of oxygen into the bulk liquid and to reach a suitable mixing. The concentration of dissolved oxygen (DO) in the liquid phase was regulated by changing the ratio of fresh air to recycled air injected in the reactor and keeping the inflow constant. The aeration flow was kept constant at 1.7 L min^{-1} giving a net air consumption of 2350 L d^{-1}. The duration of the operational cycles was of 3 h distributed according to the scheme described in Figure 6.1a. The hydraulic retention time (HRT) was fixed at 0.25 d with a feeding velocity of 4.4 mL min^{-1}.

a) SBR



b) SBRP

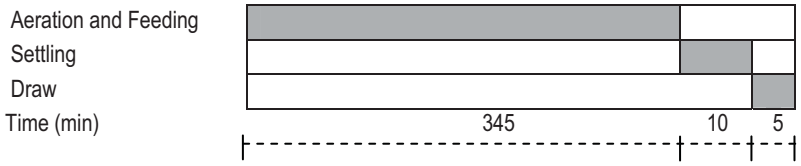


Figure 6.1. Distribution of the operational cycles of a) the granular SBR and b) the pulsing SBRP reactor.

6.3.3.2. Pulsing reactor SBRP

The SBRP was operated at room temperature (18 - 24 ºC) and the pH value ranged around 7.7 ± 0.2, both without control. The DO concentration ranged between 0.2 and 4.0 mg O_2 L^{-1} along the operational period. The DO concentration in the system was regulated by changing the air volume injected in each pulse and keeping the frequency of pulsation constant. The air volume was adjusted varying the aeration flow that ranged between 1.0 and 3.5 L min^{-1} during the experiment. The net air consumption can be obtained by multiplying the aeration flow time the pulsation frequency and this value ranged from 120 to 425 L d^{-1}. The SBRP was operated in cycles of 6 h distributed according to the scheme described in Fig. 6.1b. The hydraulic retention time was fixed at 0.5 d with a feeding velocity of 2.2 mL min^{-1}.

It is important to point out that both reactors operated in semicontinuous mode and that the feeding and aeration phases took place simultaneously during 95% of the cycle time. In both cases the DO concentration decreased to zero during the settling phase. The efficiency of the reactor was estimated by the determination of the concentrations of ammonium, nitrite and nitrate in the liquid samples collected at the end of the operational cycle. These concentrations were representative of the whole cycle, due to the length of the feeding period as it can be stated from performed cycle measurements.

6.3.3. Inocula

6.3.3.1. Granular SBR

The nitrifying granular biomass operated in this granular SBR was obtained from heterotrophic aerobic granules by the stepwise decrease of the COD/N ratio in the influent (Mosquera-Corral et al., 2005). The concentration of biomass inside the system at the beginning of this study was of 5 g VSS L^{-1} with an average diameter of the granules around 2.3 mm corresponding to the end of the operation described in Chapter 3. The reactor was carrying out partial nitrification with a DO concentration around 2 mg O_2 L^{-1} treating a synthetic wastewater with a specific ammonium oxidation rate of 0.12 g N (g VSS)$^{-1}$ d^{-1}.

6.3.3.2. Pulsing reactor SBRP

Prior to this study, the use of the pulsing sequencing batch reactor was tested to produce nitrifying granules (Belmonte et al., 2009). The reactor was inoculated with sludge from a wastewater treatment plant. After 400 days of operation, 1.07 g VSS L^{-1} of nitrifying granules with a mean diameter of 0.9 mm was accumulated in the system. The maximal nitrate production rate reached a value of 0.3 g N L^{-1} d^{-1}.

6.3.4. Operational strategy

6.3.4.1. Granular SBR

The nitrifying granular reactor was fed with the supernatant of an anaerobic sludge digester of the WWTP of Lugo (Spain) which was collected every month in 20 L containers and stored in a cold room (4 ºC). The characterization of the supernatant was: pH 7.5 – 8.3; NH_4^+ 400 – 700 mg N L^{-1}; IC (inorganic carbon) 300 – 505 mg C L^{-1} and TOC (total organic carbon) 20 – 50 mg C L^{-1}. The reactor operation was divided into different stages (Table 6.1). From Stage I to IV, the raw wastewater was diluted in a proportion 1:1 with tap water. The first stage was of adaptation to the supernatant (Stage I). During the second stage (Stage II) a stable composition of the effluent was achieved by regulating the DO concentration. Once the Anammox biomass developed in the core of the granules (Stage III), the DO was increased in order to favour the nitrogen removal. Afterwards (Stage V), the dilution ratio of the supernatant was decreased until raw sludge liquor was

used as the feeding to the reactor. The DO concentrations and the NLR were increased to study the maximal nitrogen removal rate achievable in the system and the effect of the free ammonia on the nitrite oxidizing bacteria (NOB). Finally, during Stage VI, the DO concentration was decreased to avoid nitrite oxidation. The mean molar ratio alkalinity/NH_4^+ during the whole operation stage was 1.0 ± 0.2.

Table 6.1. Operational stages in the granular SBR.

Stage	Days	DO (mg O_2 L^{-1})	Influent Dilution
I	1 – 64	2.2 ± 0.4	50%
II	65 – 120	2.7 ± 0.3	
III	121 – 243	2.7 ± 0.5	
IV	244 – 287	3.5 ± 0.5	
V	288 – 360	3.5 to 4.6	50% to 0%
VI	361 – 400	2.8 ± 0.5	0%

6.3.4.2. Pulsing reactor SBRP

The SBR was firstly fed with a synthetic autotrophic media for 79 days and then with the effluent from the anaerobic sludge digester of the WWTP of Lugo (Spain). The synthetic media was composed by 0.018 g KH_2PO_4 L^{-1}, 0.047 g K_2HPO_4 L^{-1}, 0.012 g $MgSO_4$ L^{-1}, 0.005 g KCl L^{-1}, 2 g $NaHCO_3$ L^{-1}, 0.15 g NH_4^+-N L^{-1} as ammonium chloride and 0.5 mL L^{-1} of a trace solution. The composition of the trace solution was in g L^{-1}: 1.50 $FeCl_3 \cdot 6\ H_2O$, 0.15 H_3BO_3, 0.15 $CoCl_2 \cdot 6\ H_2O$, 0.12 $MnCl_2 \cdot 4\ H_2O$, 0.12 $ZnSO_4 \cdot 7\ H_2O$, 0.06 $NaMoO_4 \cdot 2\ H_2O$, 0.03 $CuSO_4 \cdot 5\ H_2O$ and 0.03 KI. The industrial wastewater was diluted with tap water 1:1 to reach concentrations of ammonium that ranged from 0.15 to 0.35 g NH_4^+-N L^{-1}.

The reactor was operated in five different operational stages to test the effect of DO concentrations on the reactor performance (Table 6.2). Regarding the cycle operation the DO concentration remained practically constant during the aeration period.

Table 6.2. Operational stages conditions in the SBRP.

Stage	Days	DO (mg O_2 L^{-1})	Observations
I	0-54	3.1 ± 1.1	Synthetic feeding
II	55-79	2.4 ± 0.8	
III	80-124	2.4 to 0.5	Day 95: Feeding change to supernatant
IV	125-179	0.5 ± 0.1	Day 125: Anammox inoculation
V	180-350	0.3 ± 0.2	Day 180: System destabilization

In addition, on day 125, the reactor was inoculated with 0.16 g of volatile suspended solids (VSS) of biomass from another reactor (representing the 8% w/w of the total biomass amount present in the reactor and a concentration of 0.1 g VSS L^{-1}) with an Anammox activity of 0.05 g N g VSS^{-1} d^{-1} at 25 ºC.

6.3.5. Analytical methods

The concentration of VSS, the sludge volumetric index (SVI_5) and the settling velocity were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Nitrite and nitrate concentrations were determined by capillary electrophoresis (Vilas-Cruz et al., 1994). Concentrations of TOC and IC were measured with a Shimadzu analyser (TOC-5000). 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) provided with a digital camera (Coolsnap, Roper Sicientific Photometrics). For the digital image analysis the programme Image Pro Plus was used.

In order to identify bacterial populations of ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and Anammox bacteria, biomass samples were collected from each reactor. The granules were kept in their aggregated form or disaggregated. All the samples were fixed according to (Amann et al., 1995) with 4% paraformaldehyde solution. Entire granules were embedded in OCT reagent (Tissue-Tek; Miles, Ind.) prior to their cryosectioning at -35°C. Slides with a thickness of 14 μm were cut at -16 °C, and these single sections were placed on the surface of poly-L-lysine coated microscopic slides. Hybridization of all the samples was performed at 46 ºC for 90 minutes adjusting formamide concentrations at the percentages shown in Table 6.3. The used probes for in situ hybridization were 5' labelled with the fluorochromes FLUOS or Cy3 or Cy5. Fluorescence signals were recorded with an acquisition system (Coolsnap, Roper Sicientific Photometrics) coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany). A TCS-SP2 confocal laser scanning microscope (Leica, Germany), equipped with a HeNe laser for detection of Cy3 and Cy5 and one Ar ion laser for detection of FLUOS, was used with the sliced samples. Further information about the analytical methods is provided in Chapter 2.

Table 6.3. Targeted organisms and the corresponding formamide (FA) percentages for the used oligonucleotide probes.

Probe[a]	Probe sequence (5'→3')	FA (%)	Targeted organisms
EUB338	GCT GCC TCC CGT AGG AGT	0-50	*Bacteria* domain
EUB338 II	GCA GCC ACC CGT AGG TGT	0-50	Planctomycetales
EUB338 III	GCT GCC ACC CGT AGG TGT	0-50	Verrucomicrobiales
ALF1b	CGT TCG YTC TGA GCC AG	20	α-proteobacteria, some δ-proteobacteria, Spirochaetes
BET42a[b]	GCC TTC CCA CTT CGT TT	35	β- *Proteobacteria*
GAM42a	GCC TTC CCA CAT CGT TT	35	γ- *Proteobacteria*
Nso190	CGA TCC CCT GCT TTT CTC C	55	Ammonio-oxidizinjg β- *Proteobacteria*
NEU653[b]	CCC CTC TGC TGC ACT CTA	40	Most halophilic and halotolerant *Nitrosomonas* spp.
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	50	most members of the phylum *Nitrospirae*
NIT3[b]	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.
PLA46	GAC TTG CAT GCC TAA TCC	30	Planctomycetales
Amx820	AAA ACC CCT CTA CTT AGT GCC C	40	Anaerobic ammonium-oxidizing bacteria Candidatus "Brocadia anammoxidans" and Candidatus "Kuenenia stuttgartiensis"

[a]Details on oligonucleotide probes are available at probeBase (Loy et al., 2007).

[b]Used with an equimolar amount of corresponding unlabelled competitor oligonucleotide probe.

6.3.6. Calculations

6.3.6.1. Nitrogen removal rates

Ammonium and nitrite oxidation rates (AOR and NOR, respectively) and nitrogen removal rate by Anammox bacteria (ANR) in the reactors were estimated as g N L^{-1} d^{-1} based on nitrogen balances and the stoichiometry of the Anammox process (Eq. 6.2—6.5).

$$\Delta N = (NH_4^+ - N_{inf}) - ((NH_4^+ - N_{eff}) + (NO_2^- - N_{eff}) + (NO_3^- - N_{eff})) \quad (6.2)$$

$$AOR = \frac{(NH_4^+ - N_{inf}) - (NH_4^+ - N_{eff}) - \frac{\Delta N}{2.04}}{HRT} \quad (6.3)$$

$$NOR = \frac{(NO_3^- - N_{eff}) - \frac{0.26\ \Delta N}{2.04}}{HRT} \quad (6.4)$$

$$ANR = \frac{\Delta N}{HRT} \quad (6.5)$$

being HRT the hydraulic retention time (d), NH_4^+-N_{inf} the ammonium concentration in the influent (mg N L^{-1}) and NH_4^+-N_{eff}, NO_2^--N_{eff}, NO_3^--N_{eff} the ammonium, nitrite and nitrate concentrations in the effluent (mg N L^{-1}), respectively.

6.3.6.2. Observed yield

The biomass production during an interval of time was estimated using the observed yield that can be obtained according to Eq. 6.6.

$$Y_{obs} = \frac{Y}{1 + b \cdot SRT} \quad (6.6)$$

being Y the stoichiometric yield (g VSS (g N)$^{-1}$), and b the decay rate constant (d^{-1}), these values for AOB, NOB and Anammox bacteria were taken from Hao et al., (2002). SRT was the sludge retention time (d).

6.3.6.3. Total surface of the granules

The total surface of the granules (S (m^2)) was calculated according to Eq. 6.7.

$$S = \frac{V_R\ X_R}{\rho_{granule}} \frac{4\ \pi\ R_m^{\ 2}}{\frac{4}{3}\ \pi\ R_m^{\ 3}} \quad (6.7)$$

being V_R the reactor volume (L), X_R the biomass concentration in the reactor (g VSS L^{-1}), $\rho_{granule}$ the granules density (g VSS $(L_{granule})^{-1}$) and R_m the mean radius of the granule (m).

6.3.6.4. Free ammonia (FA) and free nitrous acid (FNA)

The concentrations of free ammonia and free nitrous acid were calculated according to the equilibrium between the ionized and unionized species as proposed by Anthonisen et al., (1976) (Eq. 6.8 and 6.9).

$$C_{NH_3} = \frac{C_{NH_4^+}}{\frac{e^{\frac{6334}{273+T}}}{10^{pH}} + 1} \quad (6.8)$$

$$C_{HNO_2} = \frac{C_{NO_2^-}}{10^{pH}\ e^{\frac{-2300}{273+T}}} \quad (6.9)$$

Being C_{NH4}; C_{NH3}; C_{NO2} and C_{HNO2} the concentrations of ammonium, free ammonia, nitrite and free nitrous acid (mg N L^{-1}), T the temperature (ºC) and pH the reactor pH.

6.4. Results and discussion

6.4.1. Nitrogen removal

6.4.1.1. Granular SBR

Regarding the removal of nitrogen compounds, during Stage I, fluctuations of the ammonium oxidation efficiency were observed (Fig. 6.2a) and attributed mainly to the change in the feeding composition caused by the introduction of the anaerobic supernatant, as it was indicated in Chapter 3 the reactor was previously fed with synthetic wastewater. By adjusting the DO concentration during Stage II (Fig. 6.2b), stable partial nitrification was reached between days 60 and 120 with a NO_2^-/NH_4^+ molar ratio of 1.1 ± 0.2; the concentrations of both ammonium and nitrite in the effluent were around 140 mg N L^{-1}. The ammonium oxidation rate remained stable with a nitrite production around 0.6 g NO_2^--N L^{-1} d^{-1} during this stage whereas nitrite oxidation was neglected.

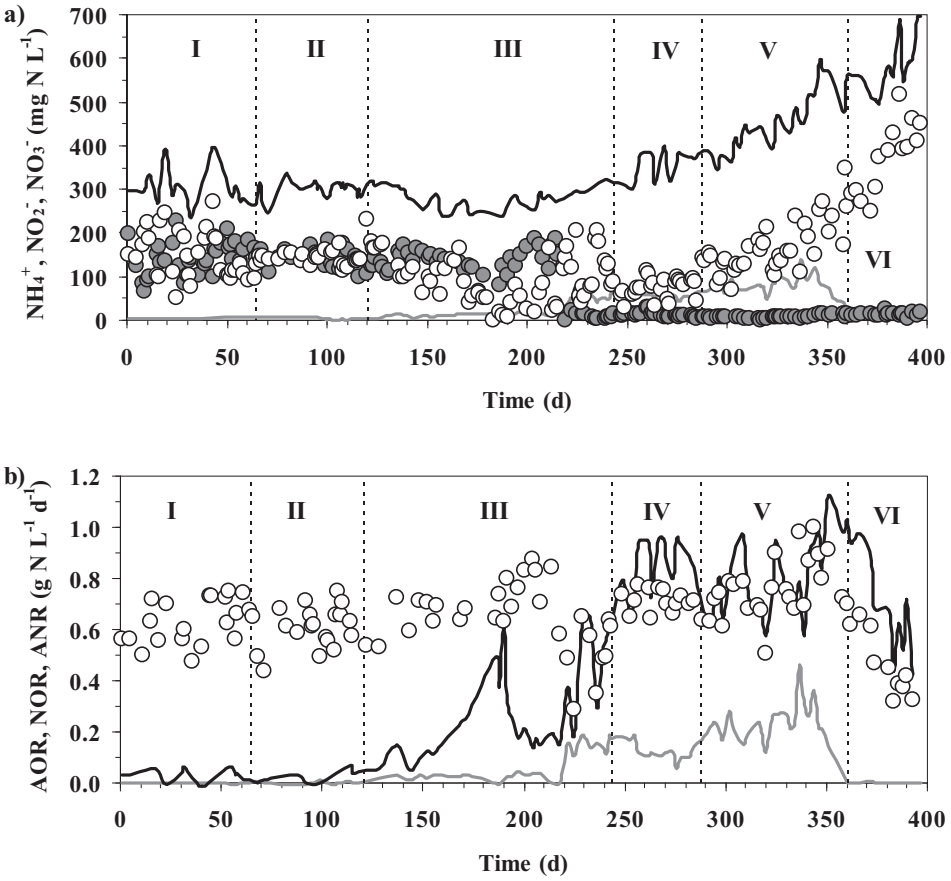


Figure 6.2. Granular SBR operation a) Concentrations of nitrogen compounds in the influent: ammonium (▬) and in the effluent: ammonium (○), nitrite (●) and nitrate (▬). b) Ammonium oxidation rate (AOR, ○), nitrite oxidation rate (NOR, ▬) and, nitrogen removal rate by Anammox bacteria (ANR, ▬).

From day 120 on, increasing nitrogen losses were registered in the granular SBR with a simultaneous change in the colour of the biomass to reddish. After an exponential increase in the nitrogen removal rate during Stage III (day 190, Fig. 6.2b), a problem with the feeding pump caused an important decrease in the ANR down to 0.2 g N L^{-1} d^{-1}. One month later, the system recovered its efficiency and an ANR of 0.6 g N L^{-1} d^{-1} was achieved at the end of this stage. Applied ALR to the system was increased from 1.2 to 1.5 g N L^{-1} d^{-1} during Stage IV. Nitrite was almost not detected in the effluent but ammonium concentrations between 30 and 120 mg NH_4^+-N L^{-1} still remained in the effluent. In spite of the increase of the DO up to 3.5 mg O_2 L^{-1} during this stage the ammonium oxidation was the limited step of the CANON process. This result agrees with the results reported by Sliekers et al., (2003) working with the CANON process in an airlift reactor. An ANR of 1.0 g N L^{-1} d^{-1} was achieved and the nitrogen removal percentage was around 60%.

During Stage V the applied ALR and the DO concentration were progressively increased up to 2.2 g N L^{-1} d^{-1} and 4.6 mg O_2 L^{-1}, respectively. Both AOR and ANR remained in similar values to those obtained during Stage IV and only an increase of the NOR from 0.1 to 0.3 g NO_3^--N L^{-1} d^{-1} was observed. This fact caused that ammonium concentration in the effluent increased significantly. Finally, DO concentration was restored to 2.8 mg O_2 L^{-1} in order to avoid nitrite oxidation (Stage VI). This action completely stopped nitrite oxidation but also caused a strong decrease of the AOR to 0.4 g N L^{-1} d^{-1}.

6.4.1.2. Air pulsing SBRP

The SBRP reactor was operated for 400 days (Belmonte et al., 2009) previously to the 350 operation days of the present work. A synthetic media was used as feeding media in order to better control the operation of the pulsing reactor since no information is available about nitrification in aggregates formed in this kind of reactors. During the first 54 days (Stage I, Table 6.2), 0.25 g N L^{-1} d^{-1} of ammonium were oxidized to nitrate operating the reactor at a mean DO concentration in the liquid media of 3.1 mg O_2 L^{-1} (Fig. 6.3a). From this point and in order to achieve partial nitrification in the system, the DO concentration was diminished by stepwise decreasing the air flow supplied in each pulse. From days 55 to day 79 the mean DO concentration was fixed at 2.4 mg O_2 L^{-1}. Under these conditions, no complete nitrification was achieved and nitrite accumulated in the liquid media due to DO limitation of NOB which present lower oxygen affinities for oxygen than AOB. From day 80 onwards the DO concentration was gradually decreased reaching a value of 0.5 g O_2 L^{-1} at the end of Stage III. During this Stage III, on day 95 of operation, the synthetic media was substituted by the supernatant of an anaerobic digester in order to prove that the system was able to operate in stable conditions treating this effluent. At the end of the Stage III, partial nitrification with 1:1 molar ratio of NH_4^+/NO_2^- was achieved while nitrate was absent due to NOB oxygen limitation. On day 125 of operation (Stage IV), once suitable conditions to grow Anammox bacteria (low DO concentration and equal concentrations of ammonium and nitrite) were achieved, 100 mL of sludge containing Anammox bacteria were inoculated to the reactor. One month after inoculation, significant decrease of ammonium and nitrite concentrations together with the slight increase of nitrate concentration and disappearance of nitrogen from the balance were measured in the reactor indicating the occurrence of an incipient Anammox activity. At this point it was considered that the nitrogen removal via the CANON process was established. To favour the nitrogen removal the nitrite concentration in the liquid media was maintained close to zero, in order to avoid nitrite inhibition of Anammox bacteria, while the volume of air injected in each pulse was slightly increased to augment the ammonium oxidation to nitrite. The increase of air volume pulsed did not revert on an increase of DO concentration in the liquid media because of its fast consumption by the ammonium oxidizing bacteria. At the end of Stage IV, a stable ANR of 0.25 g N L^{-1} d^{-1} was achieved (Fig. 6.3b).

On day 180 (Stage V), the feeding pump did not work properly and provoked the destabilization of the system due to ammonium limitation which caused DO increase and subsequent nitrite peak that reached 50 mg N L^{-1}. After 20 days the previous operational conditions were restored in the reactor and the nitrogen removal capacity increased continuously until the end of the experiment. From days 295 to 350, a stable operational period for the CANON process was obtained with an average ANR of 0.36 g N^{-1} L^{-1} d^{-1} (Fig. 6.3b) and nitrogen removal percentage of 77%. The assumption made in the balances (Eq. 6.3 to 6.5) not considering heterotrophic denitrification was justified by the low TOC removal of only 82 mg TOC L^{-1} d^{-1} which did not justify the amount of nitrogen removed from the reactor. Once nitrogen removal percentages close to the theoretical maximum value of 89% (according to the Anammox stoichiometry, Eq. 6.1) were reached, no further study of the maximal ANR reachable in this system was performed. The obtained nitrogen removal efficiency demonstrates that the growth of NOB is avoided due to nitrite and oxygen limitations.

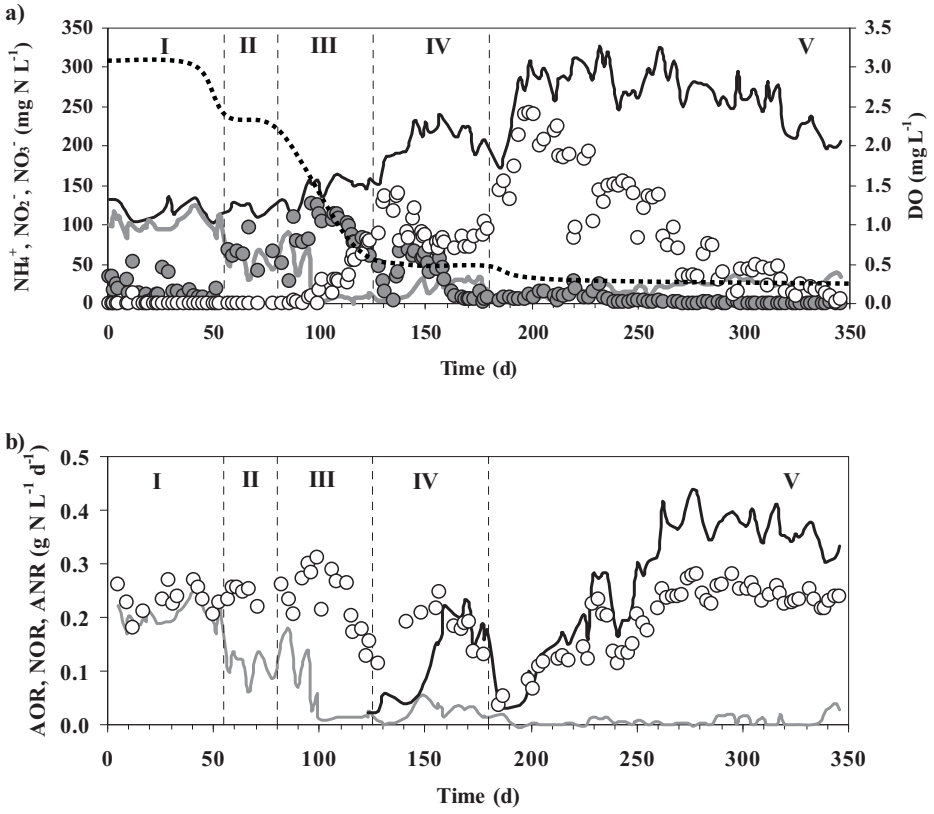


Figure 6.3. SBRP operation a) Concentrations of nitrogen compounds, in the influent: ammonium (—) and in the effluent: ammonium (○), nitrite (●) and nitrate (—).DO concentration in the bulk liquid (•••). b) Ammonium oxidation rate (AOR, ○), nitrite oxidation rate (NOR, —) and, nitrogen removal rate (ANR, —).

6.4.2. Biomass physical properties

6.4.2.1. Granular SBR

The biomass concentration in the granular SBR reactor ranged during the whole operational period between 5 and 8 g VSS L^{-1} (Fig. 6.4). During partial nitrification stages (Stages I and II), the biomass concentration was maintained around 5 g VSS L^{-1}. This value increased once the CANON process developed (from day 120 on) presumably due to the growth of Anammox biomass inside the granules (Stages III to VI), reaching a maximum concentration of 8 g VSS L^{-1} which remained stable after day 300 of operation. A similar trend was registered in the evolution of the solids concentration in the effluent which increased from a mean value of 40 mg VSS L^{-1} during the partial nitrification operation to values up to 80 mg VSS L^{-1} during the CANON operation. The SRT in the SBR varied in the range between 20 and 80 d. A slight decrease in the SRT for the last stages was associated to the increase in the VSS concentration of the effluent (Fig. 6.5d).

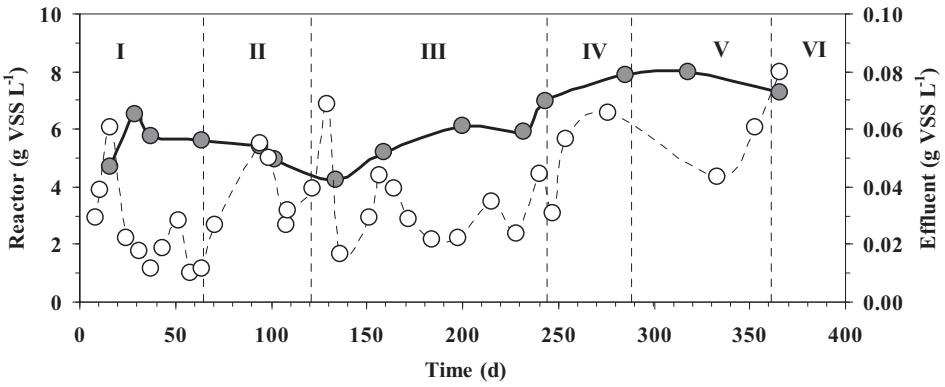


Figure 6.4. Concentrations of biomass in the granular SBR (●) and in the effluent (○).

An increase in the mean diameter of the granules during the reactor operation from 2.3 to 3.2 mm was registered. This evolution was also associated to the development of Anammox biomass inside the granules (Fig. 6.5). These values are higher than those reported for nitrifying granular systems (Campos et al., 2000; Tsuneda et al., 2003; Kim and Seo, 2006) and similar to those reported for aerobic granules grown on carbon sources (de Kreuk et al., 2005). The colour of the granules changed once the Anammox biomass developed in their inner core. The typical reddish colour of Anammox bacteria could be easily recognized when observing the granules on a stereomicroscope (Fig 6.5b).

The SVI_5 value was kept always under 50 mL (g VSS)$^{-1}$ and the settling velocity of the granular sludge ranged from 70 to 150 m h^{-1} during the whole operation, maintaining, therefore excellent settling properties.

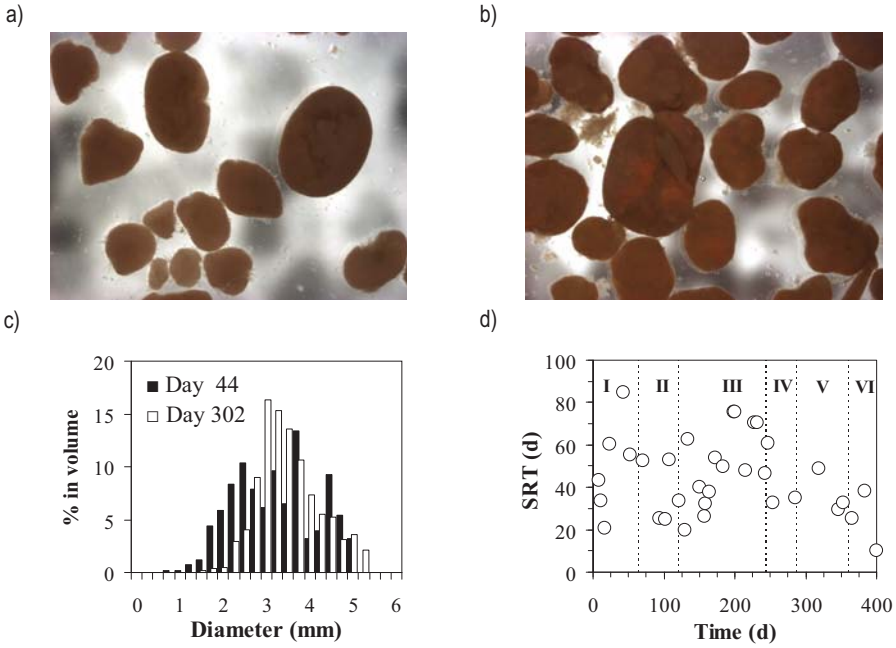


Figure 6.5. Pictures of the granular biomass of the SBR (zoom: 6.5) taken a) on day 44 and b) on day 302 and c) size distribution by volume on the cited days and d) Evolution of the SRT in the SBR.

6.4.2.2. Air pulsing SBRP

In the case of the air pulsing SBPR during the two first stages, the biomass concentration in the reactor was of 1.5 g VSS L^{-1} and solids concentration in the effluent was of 5 mg VSS L^{-1} (Fig. 6.6) which allowed working at a solids retention time (SRT) of 150 d (Fig. 6.7d). Solids concentration in the effluent increased up to 20 mg VSS L^{-1} during Stage III whereas the biomass concentration in the reactor remained constant, reducing the SRT down to 40 d. This concentration in the effluent remained constant until the end of the operation and due to the increase of biomass concentration up to 4.5 g VSS L^{-1} (Stage V) the initial value of SRT was restored (Fig. 6.7d).

Biomass production during Stage V was calculated taking into account the nitrogen consumption and the observed yield coefficients under these operational conditions (Eq. 6.6). The estimated amounts of biomass produced corresponding to AOB, NOB and Anammox bacteria were 1.27; 0.01 and 3.64 g VSS, respectively for the whole stage, which involved a retention capacity of 87% of generated biomass. A high biomass retention capacity is a key factor for a fast start-up of bioreactors working with slow growing biomass such as ammonium oxidizing and Anammox bacteria.

A significant change in size, colour and aspect of the biomass was observed from Stage I to Stage V (Fig. 6.7). During the two first stages, the biomass was composed by granules with a mean diameter of 0.8 mm. After the decrease in the DO concentration, AOB formed fluffy structures in order to overcome diffusion limitation into the granules. As the ANR increased after the inoculation, an increase in the average feret diameter of the granules was also observed reaching values around 1.6 mm, doubling the initial size, with

some granules reaching 3.6 mm of diameter. This increase could be attributed to the development of granular Anammox biomass during this stage.

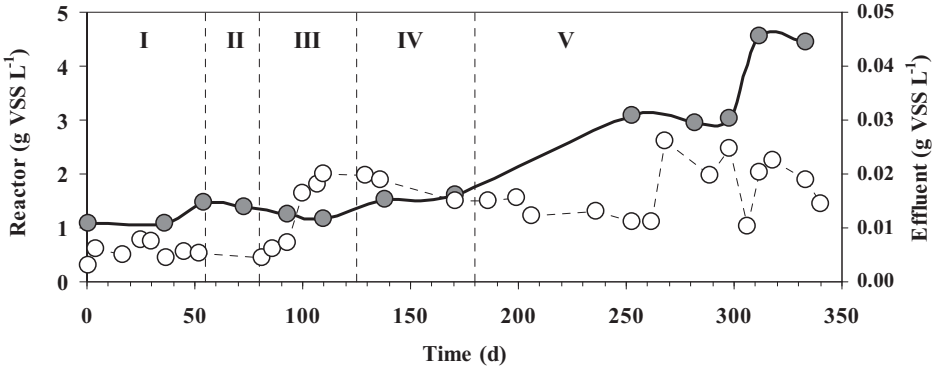


Figure 6.6. Concentrations of biomass in the SBRP (●) and in the effluent (○).

Therefore, according to Nielsen et al., (2005), two different biomass types are expected to perform the CANON process, a fluffy structure performing mainly partial nitrification and granules where a thin layer of AOB consumes oxygen protecting and providing nitrite to Anammox bacteria responsible of the autotrophic nitrogen removal.

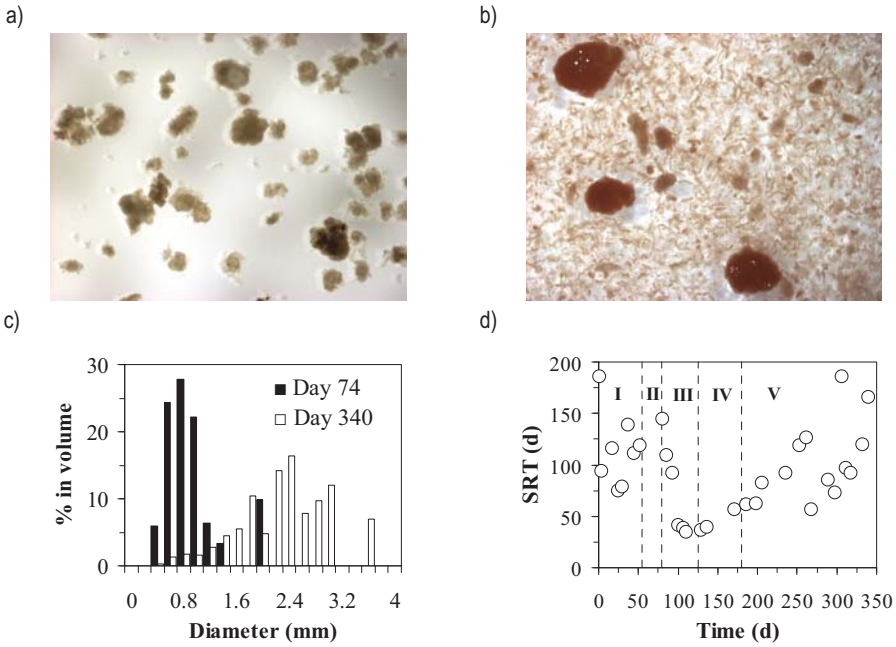


Figure 6.7. Pictures of the biomass of the SBRP (zoom: 6.5) taken a) on day 74 and b) on day 340 and c) size distribution by volume on the cited days and d) Evolution of the SRT in the SBRP.

6.4.3. Identification of bacteria populations by FISH

By the application of the FISH technique to a biomass sample collected from the granular SBR during Stage II (day 100), bacteria belonging to the genus *Nitrosomonas* were identified as the dominant AOB population in the samples. No positive results were obtained when the probes NIT3 and Ntspa712 were applied indicating the absence of nitrite oxidizing bacteria.

In order to confirm the results obtained applying mass balances during Stage VI a sample of granules was collected on day 380. The granules were sliced and pictures after applying the FISH technique were taken to confirm the presence of AOB and Anammox bacteria. Bacteria belonging to the genus *Nitrosomonas* (NEU653) were identified as the dominant AOB population in the samples and they were located in the outermost layers of the granules. Anammox bacteria gave positive results with Amx820 probes, indicating the presence of *Candidatus* Brocadia anammoxidans and/or *Candidatus* Kuenenia stuttgartiensis located in more internal layers of the granules, where oxygen was consumed by AOB. In Fig. 6.8, the zone where both bacteria coexist is shown. This is a zone where the DO concentration would be close to zero. Since AOB are producing nitrite which is consumed by Anammox bacteria, a straight relation between them is established.

This layers configuration could explain the robustness of this process. As it was already reported by Pynaert et al., (2004), the autotrophic nitrogen removal in one stage could be developed in a rotating disk contactor or in a granular SBR which confer to the Anammox process high resistance to temperature, pH or oxygen changes. This effect can be caused by the protection provided by the oxic layer with a high density of *Nitrosomonas* hindering and lowering the variations registered in the bulk liquid.

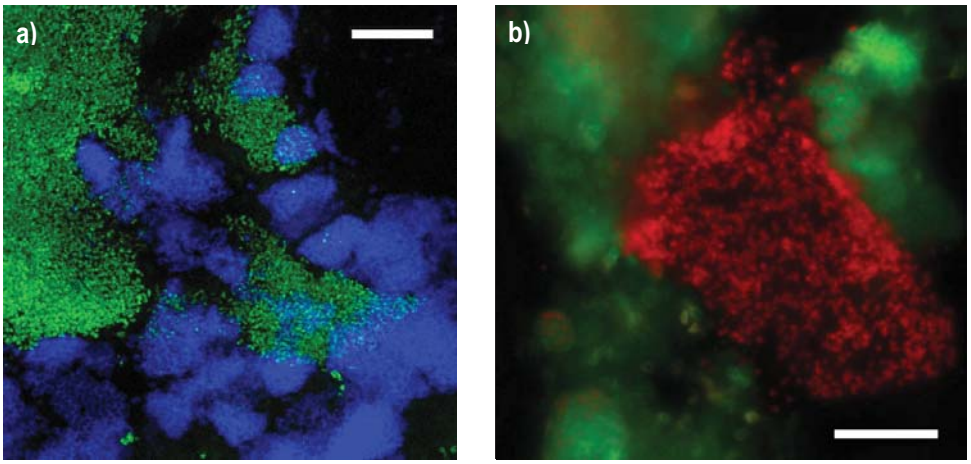


Figure 6.8. a) Image of the biomass in the granular SBR on day 380 with the applied FISH probes: NEU653 (green, FLUOS) and Amx820 (blue, Cy5). b) Image of the biomass in the SBRP on day 262 with the applied FISH probes: NEU653 (green, FLUOS) and Amx820 (red, Cy3). The bar corresponds to 15 μm.

Very similar results were obtained for the SBRP. In order to identify the main bacteria populations present in the reactor, the FISH technique was applied to biomass samples collected on day 262. By the application of FISH probes, bacteria belonging to the genus *Nitrosomonas* (NEU653) were identified as the dominant population in the samples accounting for the 75% ± 10% of the total biomass. Anammox bacteria gave positive results of both PLA46 and Amx820 probes, indicating the presence of *Candidatus* Brocadia

anammoxidans and/or *Candidatus* Kuenenia stuttgartiensis. The coexistence of ammonium oxidizing and Anammox bacteria was well-supported by the results obtained with CANON process inside the pulsing SBR.

Positive results were obtained with probes ALF1b and GAM42a, indicating the presence of some α- and γ-Proteobacteria whereas no positive results were obtained when the probes NIT3 and Nitspa712 indicating the absence of NOB.

6.4.4. Partial nitrification and Anammox growth in the reactors

As it is shown in Fig. 6.2 (Stage II of operation in the SBR) and Fig. 6.3 (at the end of the Stage III of operation in the SBRP) partial nitrification was achieved in both systems, SBR and SBRP, with the regulation of the DO concentration in the bulk liquid. The oxygen mass transfer limitation in the granules could explain the occurrence of the partial nitrification with an efficiency of 50% (as aforementioned in Chapter 3) and this fact will be analyzed in this section. No significant effect of the large SRT values measured in both reactors (Fig 6.5b and 6.7b) was observed even though these values were long enough to establish the NOB activity when neither inhibition nor substrate limitation was present.

The SHARON process is one of the technologies proposed to achieve the partial nitrification previously to the application of the Anammox process and it was successfully used at full scale to treat the effluent from sludge digesters (van Dongen et al., 2001; van der Star et al., 2007). However when the temperature of the treated effluent is lower than 24 ºC the maximal growth rate of AOB turns lower than that of NOB and ammonium is fully oxidized into nitrate (Fux et al., 2002). Therefore, to achieve partial nitrification at temperatures lower than 24 ºC other strategies, such as inhibition of NOB by NH_3 (FA) or HNO_2 (FNA) (Kim and Seo, 2006) or operation at low DO concentrations (Blackburne et al., 2008), should be applied.

Results from the granular SBR indicated that nitrite was not oxidized into nitrate until day 215 (Fig. 6.2b) in spite of the concentration of FA inside the system remained close to zero (Fig. 6.9a) while the highest rate of nitrite oxidation was registered during Stage V when FA concentrations were between 5 and 10 mg NH_3-N L^{-1}, values that would inhibit completely NOB activity according to Anthonisen et al., (1976). Therefore, there is not a direct correlation between the presence of free ammonia and the accumulation of nitrite in the system. The appearance of the activity of the NOB occurred simultaneously with an increase of the total surface of the granules due to both factors, the increase of the diameter of the granules and biomass concentration at the end of Stage III (Fig. 6.9a). This fact could indicate that partial nitrification was due to DO limitation by the external mass transfer resistance which provoked that the DO concentration on the surface of the granule was lower than that in the bulk liquid. This mass transfer resistance together with the internal one would explain that partial nitrification in granular and biofilm systems was observed at higher DO concentrations than in the case of activated sludge systems (Ruiz et al., 2003; Yun and Kim, 2003; Wyffels et al., 2004).

The oxygen limitation was even more pronounced in the SBRP since once the DO concentration in the bulk liquid decreased under 1 mg O_2 L^{-1} (after day 100) no more NOB activity was registered. At the end of Stage V (from day 270 to the end of the operation), even with free ammonia concentrations as low as 0.5 ± 0.5 mg N L^{-1} and the high surface available for oxygen transfer into the granule no nitrite oxidation took place (Fig. 6.9b).

Mass transfer resistance caused that oxygen could only penetrate around 100-400 μm into the granule (Tsuneda et al., 2003; Chiu et al., 2007) meaning that the majority of the volume of the granule was not penetrated by oxygen and, therefore, its specific aerobic activity was lower in comparison with the activated sludge systems. This drawback is compensated by the large biomass amounts retained inside the system due

to its excellent settling properties. The low biomass concentrations in the effluent of the nitrifying granular reactor would minimize the presence of solids in the influent of the Anammox SBR that could enhance heterotrophic activity compromising the process efficiency (Lackner et al., 2008). Partial nitrification with biomass in suspension controlling the DO concentration in the bulk liquid could turn unstable due to the AOB wash out and the NOB growth (Blackburne et al., 2008). In this aspect, the granular systems showed a stable value of the AOR during whole operational period and NOB activity could be avoided by decreasing DO concentration of the liquid bulk.

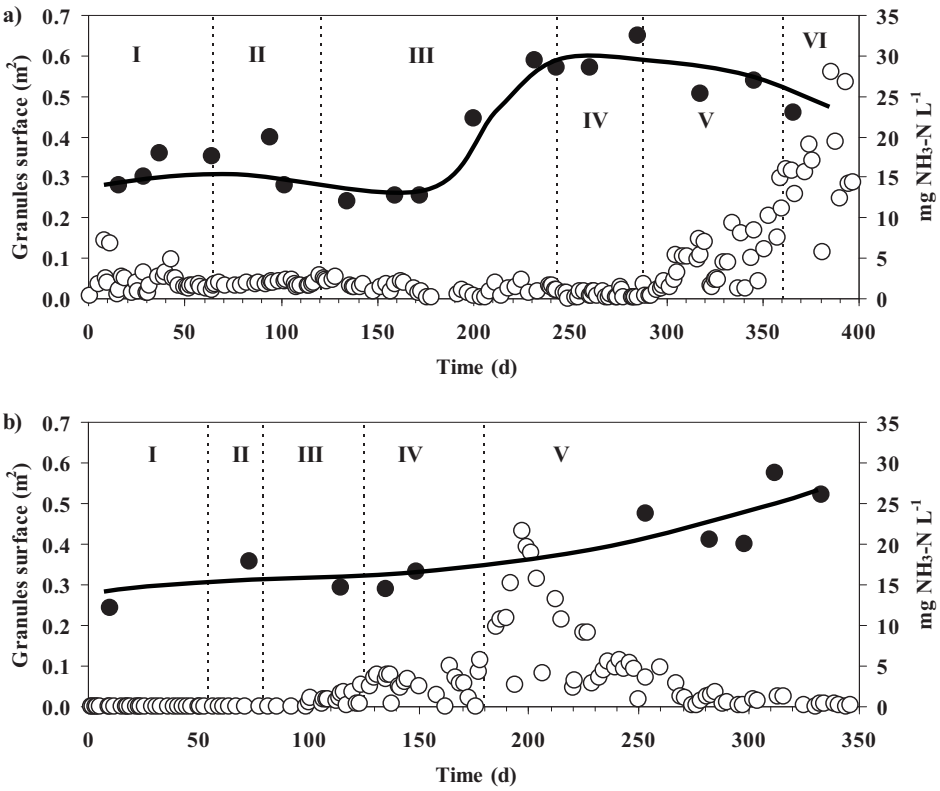


Figure 6.9. Concentrations of free ammonia (○) and total surface of the granules with trend line (●) in a) the SBR and b) the SBRP.

After day 120, a progressive increase of nitrogen loss was measured in the effluent of the granular SBR indicating the growth of Anammox biomass (and, therefore, the development of the CANON process), in spite of the existence of adverse environmental conditions for these microorganisms: presence of oxygen, high nitrite concentrations and low temperature. The same phenomenon was already reported by Siegrist et al. (1998) in a nitrifying rotating contactor. This is also in accordance to the simulation results of Hao and van Loosdrecht (2004) who stated that the natural development of the CANON process in stable nitrifying biofilms operated at low temperatures is possible. ´

Since Anammox developed spontaneously in the SBR without any inoculation, it is difficult to compare the start up of the autotrophic nitrogen removal process with other systems; however, this comparison can be performed with the data obtained from the SBRP.

6.4.5. Comparison of the start up strategies of CANON reactors

Several strategies have been tested to start-up and optimize the performance of reactors where autotrophic nitrogen removal takes place (Table 6.4). Among them, two can be pointed out: 1) to inoculate an Anammox reactor with nitrifying biomass and to supply air to maintain microaerobic conditions (Sliekers et al., 2002) or 2) to operate a nitrifying reactor under oxygen limited conditions to obtain the desired ammonium to nitrite molar ratio inside the system and then inoculate Anammox biomass (Pynaert et al., 2004; Gong et al., 2007).

Table 6.4. Comparison of several strategies to start up the autotrophic nitrogen removal process.

Background	Inoculation	Initial ANR (g N L^{-1} d^{-1})	Final ANR (g N L^{-1} d^{-1})	Start-up time	Reference[a]
Anammox reactor	Nitrifying sludge 0.2 g VSS L^{-1}	8.9	1.5	0 d	[1]
Nitrifying granules	Anammox 0.12 g VSS L^{-1}	0.05	0.25	35 d	This study SBRP
Nitrifying Biofilm	Anammox 1L	0.04	0.63	40 d	[2]
Glanerland (400 m^3) Nitritation/denitritation	Anammox from Strass WWTP 1.25 g VSS L^{-1}	0.15	0.63	55 d	[3]
Nitrifying Biofilm	Anaerobic granular sludge 10 g L^{-1}	~0	0.4	100 d	[4]
			1.7	200 d	
Nitrifying/denitrifying activated sludge	-	~0	0.36	1 year	[5]
Strass (500m^3): Nitritation/denitritation	Stepwise enrichment 4 L – 0.3 m^3 – 2.4 m^3 – 500 m^3	~0	0.7	2.5 years	[3]

[a][1] Sliekers et al., (2003) [2] Gong et al., (2007) [3] Wett, (2007) [4] Pynaert et al., (2004) [5] Gaul et al., (2005)

As represented in Table 6.4, the second strategy seems to be more suitable because an important decrease of the Anammox activity is observed when the first strategy is applied (Sliekers et al., 2003). The inoculation of Anammox enriched biomass in a nitrifying reactor accelerates the start-up process and allows registering important increases in the ANR after one or two months instead of several months or even years without inoculation. The full-scale example is very illustrative since in Strass, 2.5 years were necessary to start-up the plant in front of the 55 days registered in Glanerland once an enriched inoculum was available (Wett, 2007).

Gong et al., (2007) multiplied by 16 the ANR in 40 days whereas in our study this increase was three times lower due to the lower temperature of operation. Most of the CANON systems reported in literature were operated at temperatures around 30 ºC. It must be taken into account that, according to the activation energy of the Anammox process, the activity at 24 ºC would be only 40% of that at 35 ºC. The feasibility of Anammox systems at temperatures around 20 ºC was already reported by Isaka et al., (2007), Dosta et al., (2008) in Anammox reactors and Pynaert et al., (2004) in one stage systems.

6.4.6. Comparison of nitrogen removal performances in different reactor configurations

As Anammox microorganisms have a low growth rate, a lot of effort was focused on developing systems with biomass retention capacity in order to shorten the start up period of the process (Trigo et al., 2006) and, for this reason, most of the works were carried out under optimum conditions for the Anammox biomass (pH between 7 and 8 and temperature higher than 30 ºC). However, up to now, limited information is available on the efficiency of this process operated under low temperatures. Isaka et al., (2008) and Dosta et al., (2008) adapted respective Anammox systems by gradually decreasing the temperature and, in both cases, a strong decrease of the nitrogen conversion rate was observed. Generally, CANON systems reported in the literature were operated at 30-35 ºC (Table 6.5). In the present study, the granular SBR and the pulsing SBRP were operated at temperatures around 20 ºC, reaching rates of nitrogen removal up to 1.1 g N L^{-1} d^{-1} and 0.45 g N L^{-1} d^{-1} respectively, which are in the range of 0.075 to 1.5 g N L^{-1} d^{-1} reported for CANON systems operated at higher temperatures.

Different technologies have been used to carry out autotrophic nitrogen removal in one or two units (Table 6.5). Nitrogen removal rates achieved with both configurations are in the same range when the systems are operated at a temperature close to 30 ºC. However, in this study, the use of one single unit allowed the achievement of higher treatment capacities compared to the two units configuration, basically due to the negative effects of low temperatures on Anammox biomass and its necessity of an adaptation period. In general, the use of one single reactor could represent some advantages with respect to the two units configuration such as, lower capital costs and less oxygen consumption by AOB, which prevent possible negative effects on the Anammox bacteria. Nevertheless, the application of the two units configuration would be appropriated when toxic or organic biodegradable compounds are present in the feeding, since these compounds will be degraded in the nitrifying unit avoiding its entrance in the Anammox reactor.

Comparing now the granular SBR and the pulsing SBRP, the granular SBR presented higher ANR but also higher DO concentration in the bulk liquid and higher VSS in the reactor. The specific Anammox activity of the biomass was calculated dividing the ANR by the amount of VSS in the reactor, its maximal value was around 0.1 g N (g VSS)$^{-1}$ d^{-1} in both systems despite the lower DO concentration in the SBRP. The explanation is that the granular SBR was composed almost exclusively by granules whereas in the SBRP a mixture of granules and flocs was present. Since AOB activity is the limiting step in the reactor, despite the DO concentration is higher in the SBR, its lower specific surface availability due to the higher diameter of the granules difficult the oxygen mass transfer as aforementioned.

Therefore, comparing both reactors, it is inferred that the aeration costs in the granular SBR would be higher to achieve the same Anammox specific activity than in the pulsing SBR. On the other hand, the granular biomass of the SBR presented better settling properties than the biomass of the SBRP.

From the results obtained, it is inferred that both reactors (the granular SBR and the pulsing SBRP) could be a suitable technology to carry out autotrophic nitrogen removal at moderately low temperatures. The good retention capacity of both reactors and the low VSS concentration in the effluent will minimize the size or even the need of a posterior settler. Finally, the high H/D ratio of the reactors would reduce the surface requirement and would make the technology promising under an economical point of view. More studies are necessary to study the feasibility of a pulsing device at an industrial scale.

Table 6.5. Two units or one unit configuration systems for autotrophic nitrogen removal.

	Reactor[a]	Feeding	Volume (L)	T (ºC)	ANR (g N L^{-1} d^{-1})	N removal (%)	DO (mg O_2 L^{-1})	Reference
Two R. Config.	CSTR + SBR	Reject water		36	0.36[b]	80	N.A.[c]	van Dongen et al., 2001
		Reject water	5600	30	0.30[b]	90	2.8[d]	Fux et al., 2002
	MBR + MBR	Reject water		20 - 30	0.55[b]	82	0.2[d]	Wyffels et al., 2004
One Reactor Configuration	Airlift	Synthetic	1.8	N.A.[c]	1.5	42	0.5	Sliekers et al., 2003
	MBBR	Reject water	50	30	0.36	60	1.8	Gaul et al., 2005
		Reject water	319,000	N.A.[c]	0.25 - 0.35	70	<1	Rosenwinkel and Cornelius, 2005
		Reject water	2100	25	0.4	62	1.9	Szatkowska et al., 2007
	RBC	Synthetic	44	29	1.05	89	0.6	Pynaert et al., 2003
		Synthetic	50	30	1.80	88	0.3	Pynaert et al., 2004
		Reject water	50	14	0.42	42	1.0	Pynaert et al., 2004
	MABR	Synthetic	4	35	0.77	89	0.5	Gong et al., 2007
	UGBR	Reject water	10	30	0.06	76	N.D.[e]	Ahn and Choi, 2006
	SBR	Reject water	500,000	28	0.7	86	0.3	Wett, 2007
		Reject water	1.5	20	1.0	60	3.5	This study: SBR
		Reject water	1.5	21	0.36	78	0.5	This study: SBRP

[a] CSTR: Continuous Stirred Tank Reactor, SBR: Sequencing Batch Reactor, MBR: Membrane-assisted BioReactor, MBBR: Moving Bed Biofilm Reactor, RBC: Rotating Biological Contactor, MABR: Membrane Aerated Biofilm Reactor, UGBR: Upflow Granular Bed Reactor

[b] ANR for the two units system was calculated on the basis of the total volume of the system

[c] Data not available

[d] DO concentration in the reactor carrying out partial nitrification

[e] The liquid is not aerated in the reactor itself but in an external aeration chamber until reaching 6 mg O_2 L^{-1}

6.5. Conclusions

● The operation of a continuously aerated granular SBR or a pulsing SBR are suitable technologies to carry out partial nitrification and autotrophic nitrogen removal (CANON process) at low temperatures.

● The partial nitrification was obtained in both systems treating the supernatant of an anaerobic digester obtaining a suitable NO_2^-/NH_4^+ ratio and avoiding nitrite oxidation. Free ammonia played a secondary role and the DO concentration and the total surface of the granules were the key parameters to avoid NOB growth in the granules.

● Nitrogen losses in the granular SBR were justified by the appearance of the Anammox process leading to the development of the combined activity of the AOB and the Anammox bacteria in the same granule. Autotrophic nitrogen removal rates up to 1.1 g N L^{-1} d^{-1} were achieved in this reactor.

● In the pulsing SBRP, the strategy of inoculating Anammox biomass in a reactor running partial nitrification allowed a quick start-up of the CANON process. A high nitrifying activity in the reactor before the inoculation protected the Anammox bacteria from oxygen and provided them enough nitrite during the start-up period. Significant nitrogen removal rates around 0.25 g N L^{-1} d^{-1} were measured 35 days after inoculation of Anammox biomass. The maximum nitrogen removal rate obtained in this reactor was of 0.45 g N L^{-1} d^{-1} treating the supernatant from an anaerobic sludge digester.

● The operation of SBR allowed simple and robust regulation of the DO concentration of the liquid bulk for the stable operation of the autotrophic nitrogen removal in a single unit. The good biomass retention capacity of the reactors allowed the achievement and maintenance of adequate conditions to perform simultaneously ammonium oxidation and Anammox processes at moderately low temperatures.

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Chapter 7

Microbial community distribution and activity dynamics of granular biomass in a CANON reactor[1]

Summary

The application of microelectrodes to measure oxygen and nitrite concentrations inside CANON granules operated at 20 ºC and the application of FISH technique to cryosectioned slices of granules showed the presence of two differentiated zones inside them: an external nitrification zone and an internal Anammox zone. Microprofiles carried out at different oxygen concentrations in the bulk liquid (from 1.5 to 35.2 mg O_2 L^{-1}) revealed that the oxygen is consumed in a range of widths varying from 100 to 350 μm with a maximal consumption rate of 80 g O_2 $(L_{granule})^{-1}$ d^{-1}. On the other hand, Anammox activity was registered in the range of depth inside the granules between 400 and 1000 μm.

FISH analysis of these layers allowed the identification of *Nitrosomonas* spp. and *Candidatus* Kuenenia Stutgartiensis as the main populations carrying out ammonia aerobic and anaerobic oxidation, respectively. The nitrogen removal capacity of the studied sequencing batch reactor containing the granular biomass was 0.5 g N L^{-1} d^{-1}. This value is similar to the mean nitrogen removal rate obtained from calculations based on in- and outflow concentrations. Information obtained in the present work allowed the establishment of a simple control strategy based on the measurements of NH_4^+ and NO_2^- in the bulk liquid and acting over the dissolved oxygen concentration in the bulk liquid and the hydraulic retention time value of the reactor.

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

7.1. Introduction

Nowadays, the removal of nutrients from wastewater is a major concern due to the strengthening of disposal normatives. Wastewaters characterized by low organic matter content and high nitrogen concentrations are difficult to be treated by conventional processes like nitrification-denitrification. In these cases the Anammox process arose as an interesting alternative to treat this type of wastewaters since these bacteria are able to perform the anaerobic oxidation of ammonium with nitrite as electron donor. As a previous step, part of the ammonium has to be oxidized into nitrite. This step can be carried out in a previous reactor, e.g., a Sharon reactor (Mosquera-Corral et al., 2005) or a granular nitrifying reactor (Vázquez-Padín et al., 2009a). Another possibility consists of performing the partial nitrification and the Anammox processes in one single reactor. This process has been given different names: CANON (Third et al., 2001), OLAND (Kuai and Verstraete, 1998) and deammonification (Hippen et al., 1997) processes. Under microaerobic conditions, ammonia oxidizing bacteria (AOB) oxidize ammonium into nitrite consuming the dissolved oxygen (DO) and creating anoxic niches where Anammox bacteria can exist and convert both ammonia and nitrite into nitrogen gas and produce small amounts of nitrate. The optimization of the performance of the autotrophic nitrogen removal in the CANON process requires the control of DO and NO_2^- concentrations. To establish an adequate control of the DO concentration in the liquid media is necessary: 1) to avoid the inhibition of Anammox bacteria caused by DO concentrations higher than 0.5% of air saturation (Strous et al., 1997), 2) to prevent the growth of nitrite oxidizing bacteria (NOB) which have lower affinity for oxygen compared to AOB. The control of NO_2^- concentration is necessary since this compound inhibits Anammox activity although variable ranges of concentrations are provided in the literature for this inhibitory effect. Dapena-Mora et al., (2007) reported that concentrations of nitrite of 350 mg NO_2^--N L^{-1} corresponded to 50% inhibition of Anammox bacteria.

Due to the slow growth of both nitrifying and Anammox bacteria involved in the CANON process, the use of good biomass retention systems is mandatory to reach significant nitrogen removal rates. In this sense, the development of granular biomass allows accumulating large biomass concentrations in the reactors without the need of carrier material. Moreover, the use of granular biomass allows the existence of substrates gradients, in such way that the external part of granule could be under aerobic conditions while anoxic conditions would maintain in the core of the granule. Therefore, several biological processes can be carried out in the same granule: partial nitrification in the outer part and Anammox process in the inner part. The potential of the granular technology to carry out autotrophic nitrogen removal has been demonstrated (Vlaeminck et al., 2008; Vázquez-Padín et al., 2009a); allowing the treatment of nitrogen loads similar to systems with two different units for partial nitrification and Anammox processes, respectively.

Generally, the operational strategy of granular CANON systems is only based on the oxygen and nitrite concentrations measured in the bulk liquid. In this sense, the knowledge of the concentration profiles of these compounds and the distribution of bacterial populations inside the granule would be very useful in order to a better understanding and control of the CANON process. Microsensors, due to their very small dimensions, can be used for the determination of substrate profiles while the distribution of bacterial populations could be determined by microbiological techniques (e.g. FISH, PCR). The combination of microbiological techniques (e.g. FISH, PCR) and measurements using microelectrodes has been used to obtain detailed analysis of the in situ structure and function of nitrifying biofilms. Some examples are:

1) To estimate kinetic parameters which can be further used in mathematical models (Schramm et al., 1999; Kindaichi et al., 2006).

2) To study the mass transport of substrates through biofilms or aggregates: determination of the limiting substrate, the active zone of the biofilm and the activities under different substrate concentrations (de Beer et al., 1993; Gieseke et al., 2003).
3) To determine the consumption rates of substrates in non homogeneous biofilm reactors (Schramm et al., 1999; Kindaichi et al., 2007).
4) To corroborate the environmental conditions (e.g., pH) inside the biofilm (Gieseke et al., 2006).
5) To observe the stratification of biomass (Okabe et al., 1999; Kindaichi et al., 2006).

Therefore, combining microelectrode measurements and FISH analysis allows gaining information about the substrate concentrations to which the different layers of the biofilm/aggregate are exposed and the microbial populations involved in the different biological processes. All this microscopic information can further be transposed to a macroscopic level, giving valuable information about the possible control strategies of CANON reactors.

7.2. Objectives

● Since little information is known from a microscopic point of view about granules performing complete autotrophic nitrogen removal and taking into account the relevance of DO and NO_2^- concentrations, the objectives of this study were: the identification of the main bacteria populations present in the granules, the determination of their distribution inside the granules and the estimation of their activities by combining the concentrations profiles measured with microelectrodes and FISH images taken from cryosectioned slices of granules. From the combination of the previously obtained results a better insight about the performance of the completely autotrophic nitrogen removal granules was obtained.

7.3. Materials and Methods

7.3.1. Reactor description

The growth of biomass in the form of granules performing the CANON process was described elsewhere (Chapter 6, Vázquez-Padín et al., 2009a). Complete nitrification to nitrate and later partial nitrification to nitrite were carried out by regulation of the DO concentration in the bulk liquid. Finally, Anammox bacteria were grown in the anoxic core of the granules to form the CANON granules.

Five months after the macroscopic evidence of Anammox activity in CANON granules (day 330, chapter 6), 4 g VSS of granular biomass from the reactor operated at the University of Santiago de Compostela were inoculated in a sequencing batch reactor (SBR) with a working volume of 0.7 L at the University of Aarhus. This new reactor was operated in cycles of 3 h distributed as: 175 min of aeration and feeding, 1 min of settling and 4 min of effluent withdrawal. The hydraulic retention time (HRT) was fixed at 0.45 d and the exchange volume was fixed at 30%.

The reactor was operated at room temperature which ranged between 19 and 22 ºC. The pH value was not controlled and ranged from 7.0 to 8.1 with a mean value of 7.6 ± 0.2. Air was supplied through a diffuser at the bottom of the reactor by using an air pump to promote the transfer of oxygen into the bulk liquid and to reach a suitable mixing. The average DO concentration in the reactor was 6.6 ± 0.5 mg O_2 L^{-1} and represented the main difference with the operational conditions from the reactor operated at the laboratory in Santiago de Compostela where the DO remained around 3.5 mg O_2 L^{-1}.

The CANON SBR was fed with the supernatant of the anaerobic sludge digester of the WWTP of Aarhus (Denmark) which was stored in a cold room (4 ºC). The composition of the supernatant was: pH of 7.6—8.3; ammonium concentration of 256—666 mg N L^{-1}; inorganic carbon (IC) of 232—374 mg IC L^{-1} and total organic carbon (TOC) of 103—150 mg TOC L^{-1}.

Figure 7.1. Photographs of the granular SBR.

7.3.2. k_La measurement

An experimental estimation of the oxygen gas-liquid transfer coefficient (k_La) was carried out 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). The value of the k_La for dissolved oxygen obtained in the reactor was of 1 min^{-1}, a similar value to the one obtained in the SBR installed in Santiago de Compostela.

7.3.3. Microscale experiments

The microelectrodes were used to measure the concentration of nitrite and dissolved oxygen at different depth positions inside the granules performing the CANON process and to obtain the corresponding microprofiles. The granules were collected directly from the CANON SBR and inserted on a needle supported on a metal grid inside the experimental chamber. In order to simulate the hydrodynamic conditions from the reactor, the aeration volume in the chamber was regulated to a value which maintained the k_La (gas-liquid mass transfer coefficient) at 1 min^{-1} which was the value obtained in the SBR. All the microprofiles were measured in granules with average diameters of 5 mm. The mean temperature of the aerated chamber was 20 ± 1 ºC. Granules were kept for 1 h inside the chamber as a pre-incubation period to create pseudo steady state conditions. Concentration profiles were recorded by introducing the sensors into the granules at different depth positions using a manual micromanipulator. A dissection microscope was used to visually estimate the position of the granule/water interface by visual observation. For each granule and experimental condition tested several microprofiles were measured (the number of microprofiles performed is described in the legend of figures as n). Microprofiles of dissolved oxygen were performed by measuring its concentration at depth intervals of 25 µm while in the case of the microprofiles of NO_2^- the measurements were performed at 50 or 100 µm due to the slower response time of this sensor. The sensor signal was continuously recorded on a strip-chart recorder.

The liquid media inside the chamber was the anaerobic digester supernatant diluted with tap water. The concentration of ammonium was maintained constant in all experiments at 140 mg N L^{-1} in order to avoid ammonium limitation in the experiments and the nitrite concentrations varied from 0.7 mg NO_2^--N L^{-1} to 42 mg NO_2^--N L^{-1} by means of $NaNO_2$ addition to the diluted supernatant. The microsensors were calibrated previous to each experiment using different nitrite concentrations in the medium.

Dissolved oxygen concentrations in bulk liquid were varied between 1.5 and 35.2 mg O_2 L^{-1}. These different DO concentrations were achieved by flushing a mixture of pure O_2 and air for DO values in the aeration chamber above air saturation or a mixture of air with N_2 for values under air saturation. In order to maintain a constant k_La, in all the experiments the total gas flow (N_2/air/O_2) was kept constant.

7.3.4. NO_2^- and O_2 microsensors

Microelectrodes were used to determine concentrations of the measured compounds inside the granules due to their small sizes.

A Clark-type O_2 microsensor equipped with a guard cathode was used for microscale analysis of DO (Revsbech, 1989). These sensors were constructed with tip diameters of 10 μm and they had 90% response time lower than 1 s. The oxygen microsensor was calibrated at two points, the zero was established dropping the microelectrode in an anoxic alkaline ascorbic acid solution and the saturation was obtained or with an air saturated solution or with pure oxygen saturated solution depending on the DO analyzed in the aeration chamber.

The NO_2^- microelectrode was a biosensor, i.e., it was based on a combination of biological reactions catalyzed by bacteria and an electrochemical N_2O microsensor. The NO_2^- microsensor consisted of an immobilized pure culture of *Stenotrophomonas nitritireducens*, which reduced NO_2^- to N_2O, coupled to a Clark type N_2O microsensor. The 90% response time was 30-50 s. Due to the broad range of NO_2^- concentrations in the bulk liquid used in the performed experiments from 0.7 mg NO_2^--N L^{-1} to 42 mg NO_2^--N L^{-1} it was necessary to add tungstate inside the biosensor in order to measure the highest NO_2^- concentrations. The tungstate increased the stability of the microsensor and slowed down the nitrite consumption rate of the bacteria (Nielsen et al., 2005).

7.3.5. Analytical methods

The pH and the concentrations of DO, ammonia, volatile suspended solids (VSS), and sludge volumetric index (SVI_5) were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Nitrite and nitrate concentrations were determined by capillary electrophoresis (Vilas-Cruz et al., 1994). Concentrations of TOC and IC were measured with a Shimadzu analyser (TOC-5000). Density of the granules was measured using the dextran blue method described by Beun et al., (2002). The morphology and size distribution of the granules were measured regularly by using an image analysis procedure with a stereomicroscope (Stemi 2000-C, Zeiss) provided with a digital camera (Coolsnap, Roper Sicientific Photometrics). For the digital image analysis the programme Image Pro Plus was used.

In order to identify bacterial populations of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and Anammox bacteria, granules from the reactor were collected, kept in their aggregated form or disaggregated, and fixed according to Amann et al., (1995) with 4% paraformaldehyde solution. Entire granules were embedded in OCT reagent (Tissue-Tek; Miles, Ind.) prior to their cryosectioning at -35°C. Slides with a thickness of 14 μm were cut at -16 °C, and these single sections were placed on the surface of poly-L-lysine

coated microscopic slides. Hybridization was performed at 46 ºC for 90 minutes adjusting formamide concentrations at the percentages shown in Table 7.1. The used probes for in situ hybridization were 5' labelled with the fluorochromes FLUOS, Cy3 or Cy5. A TCS-SP2 confocal laser scanning microscope (Leica, Germany), equipped with a HeNe laser for detection of Cy3 and Cy5 and one Ar ion laser for detection of FLUOS, was used with the sliced samples.

The method to quantify bacterial populations was based on the one published by Crocetti et al., (2002). The digital image analysis program used was Image ProPlus. For the quantification of bacteria populations, cryosectioned slices of granules were used to perform a triple hybridization with EUBmix (a mixture of EUB338, EUB338 II and EUB338 III) labelled with Cy5, Amx820 labelled with Cy3 and NEU653 labelled with FLUOS. Several pictures were taken subsequently from the granule surface throughout the active layers with the confocal microscope at a magnification of 630 times. The pictures were superimposed to represent the whole active layers and then discretized in layers of 100 μm with the help of Adobe Photoshop® software. Form each image three different colour components corresponding to each fluorochrome were separated generating three different images. The area corresponding to the fluorescence of each FISH probe was obtained as the area of all pixels above a manually determined minimum pixel intensity. In order to be able to compare the areas occupied by the different populations, the maximal area occupied in a discretized picture was taken as reference and the values obtained with the different probes in the different layers were obtained as normalized area values. Further information about the analytical methods is provided in Chapter 2.

Table 7.1. Targeted organisms and the corresponding formamide (FA) percentages for the used oligonucleotide probes.

Probe[a]	Probe sequence (5'→3')	% FA	Targeted organisms
EUB338	GCT GCC TCC CGT AGG AGT	0-50	Domain *bacteria*
EUB338 II	GCA GCC ACC CGT AGG TGT	0-50	Planctomycetales
EUB338 III	GCT GCC ACC CGT AGG TGT	0-50	Verrucomicrobiales
Nso190	CGA TCC CCT GCT TTT CTC C	55	Ammonia-oxidizing β- *Proteobacteria*
NEU653[b]	CCC CTC TGC TGC ACT CTA	40	Most of the halophilic and halotolerant *Nitrosomonas* spp.
Ntspa712[b]	CGC CTT CGC CAC CGG CCT TCC	50	Most members of the phylum *Nitrospira*
NIT3[b]	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.
Amx820	AAA ACC CCT CTA CTT AGT GCC C	40	Anaerobic ammonium-oxidizing bacteria *Candidatus* Brocadia anammoxidans and *Candidatus* Kuenenia stuttgartiensis
Kst157	GTT CCG ATT GCT CGA AAC	25	*Candidatus* Kuenenia stuttgartiensis
Ban162	CGG TAG CCC CAA TTG CTT	40	*Candidatus* Brocadia anammoxidans

[a] Details on oligonucleotide probes are available at probeBase (Loy et al., 2007).

[b] Used with an equimolar amount of corresponding unlabeled competitor oligonucleotide probe.

7.3.6. Calculations

7.3.6.1. Estimation of oxygen and nitrite consumption and production rates

Mass balances were calculated for oxygen and nitrite in the granules assuming a flat geometry was assumed to describe the shape of the granules. The activities of the processes involved in the nitrogen removal inside the granules were restricted to an external layer of 1 mm (as it will be further discussed) which was smaller than the mean radius of the granules used to record the microprofiles (mean radius of 2.5 mm).

The balance of component for a flat geometry in a solid matrix can be written according to Eq. 7.1 assuming that the compound mass transfer is carried out only by diffusion and that the granule has an homogeneous structure since the diffusivity (D) is considered constant (Lorenzen et al., 1998).

$$\frac{\partial C(z,t)}{\partial t} = D\,\frac{\partial^2 C(z,t)}{\partial z^2} + R(z) \tag{7.1}$$

where C is the concentration of compound (g L^{-1}), z the depth coordinate in the granule (dm), t the time (d) and R the reaction rate (g $(L_{granule})^{-1}$ d^{-1}). Considering steady state conditions the Eq. 7.2 is obtained.

$$D\,\frac{\partial^2 C(z)}{\partial z^2} = -R(z) \tag{7.2}$$

making A(z) = -R(z)/D and using Euler's formula for numeric integration the following Eq. 7.3 is obtained.

$$\frac{\partial C}{\partial z_{n+1}} = \frac{\partial C}{\partial z_n} + h\,A_n \tag{7.3}$$

where h represents the step size used for numerical integration. This discretization parameter was of 25 µm for oxygen profiles and of 50 µm for nitrite concentrations in the bulk liquid smaller than 2.8 mg NO_2^--N L^{-1} and of 100 µm for higher NO_2^- concentrations. After further integration Eq. 7.4 is obtained.

$$C_{n+1} = C_n + h\,\frac{\partial C}{\partial z_n} \tag{7.4}$$

Substituting Eq. 7.3 in Eq. 7.4, the Eq. 7.5 is obtained.

$$C_{n+1} = C_n + h\left(\frac{\partial C}{\partial z_{n-1}} + h\,A_{n-1}\right) \tag{7.5}$$

Using the Solver tool (available in Microsoft Excel® software) the values of A were iterated in order to minimize the error between the concentration calculated with Eq. 7.2 and that one measured with the microelectrode. By multiplying the value of A obtained in a discretization point by the diffusivity of the substrate desired, the value of R(z), i.e., the local volumetric consumption rate can be obtained. The diffusion coefficients of NO_2^- and O_2 in water at 20 ºC were chosen as $1.5 \cdot 10^{-4}$ and $1.7 \cdot 10^{-4}$ m^2 d^{-1}, respectively (Picioreanu et al., 1997).

To calculate the net fluxes of substrates (J, g N m^{-2} d^{-1}) through the diffusive boundary layer (DBL) separating the surface of the granule and the bulk liquid, the Fick's first law was used (Eq. 7.6).

$$J = -D_W\,\frac{C_b - C_s}{\delta_h} \tag{7.6}$$

being D_w the molecular diffusion coefficient in water (m^2 d^{-1}), C_b is the bulk liquid concentration (g m^{-3}), C_S is the concentration (g m^{-3}) at the surface of the granule and δ_h is the hypothetical (also called effective) diffusive boundary layer thickness (m) which is defined by extrapolating the radial oxygen gradient at the granule-water interface to the bulk water phase concentration (Ploug et al., 1997).

7.3.6.2. Nitrogen removal rates

Ammonia oxidation rates (AOR) and nitrogen removal rates by Anammox bacteria (ANR) of the CANON granular reactor were estimated as g N L^{-1} d^{-1} based on nitrogen balances and the stoichiometry of the Anammox process (1.02 moles of dinitrogen gas produced per mole of ammonium reacted).

$$\Delta N = (NH_4^+ - N_{inf}) - ((NH_4^+ - N_{eff}) + (NO_2^- - N_{eff}) + (NO_3^- - N_{eff})) \quad (7.7)$$

$$AOR = \frac{(NH_4^+ - N_{inf}) - (NH_4^+ - N_{eff}) - \frac{\Delta N}{2.04}}{HRT} \quad (7.8)$$

$$ANR = \frac{\Delta N}{HRT} \quad (7.9)$$

being, ΔN the difference between total nitrogen concentration in the influent and effluent (g N L^{-1}), NH_4^+-N_{inf} the ammonium concentration in the influent (g N L^{-1}) and NH_4^+-N_{eff}, NO_2^--N_{eff}, NO_3^--N_{eff} the ammonium, nitrite and nitrate concentrations in the effluent (g N L^{-1}), respectively.

7.3.6.3. Estimation of the number of granules in the SBR

The number of granules in the reactor was calculated as follows:

$$V_{granule} = V_R \cdot X_R / \rho_{granule} \quad (7.10)$$

$$n_T = V_{granule} / (4/3 \cdot \pi \cdot R_m^3) \quad (7.11)$$

being $V_{granule}$ the volume of granules (L), V_R the reactor volume (L), X_R the VSS concentration in the reactor (g VSS L^{-1}), $\rho_{granule}$ the granules density (g VSS $(L_{granule})^{-1}$), n_T the number of granules, R_m the average radius of the granules (dm).

In order to estimate either the AOR or the ANR from the microsopic observations, Eq. 7.12 was used to the zone were AOB or Anammox bacteria were located.

$$Rate = \frac{n_T \sum_{z=1}^{n} \left(R(z) \frac{4}{3} \pi \left(R_z^3 - R_{z-1}^3 \right) \right)}{V_R} \quad (7.12)$$

Where the rate is calculated in g $(L_{reactor})^{-1}$ d^{-1}, R(z) is the local reaction rate (g $(L_{granule})^{-1}$ d^{-1}), V_R is the reactor volume (L), and R corresponds to the radius (dm).

7.4. Results and discussion

7.4.1. Operation of the CANON SBR

The SBR was operated to keep the granular biomass performing the partial nitrification and the Anammox processes active in order to be used for the determination of the O_2 and NO_2^- profiles with the microelectrodes. The SBR reactor was operated at DO concentrations around 6.6 mg O_2 L^{-1} and at a temperature of 20 ºC. The mean NO_2^- concentration in the effluent was 25 mg N L^{-1}. The total nitrogen removal rate (Fig. 7.2) ranged between 0.35 and 0.91 g N L^{-1} d^{-1}. Those values are between the highest ones registered for autotrophic nitrogen removal in one reactor despite the low temperature of operation (in Chapter 6, a comparison of the nitrogen removal rates obtained in CANON systems can be found, Table 6.5).

According to Dosta et al., (2008) the specific activity of Anammox biomass at 37 ºC is 3.6 times higher than at 20 ºC, therefore, the operation at higher temperature would allow achieving larger biomass activities.

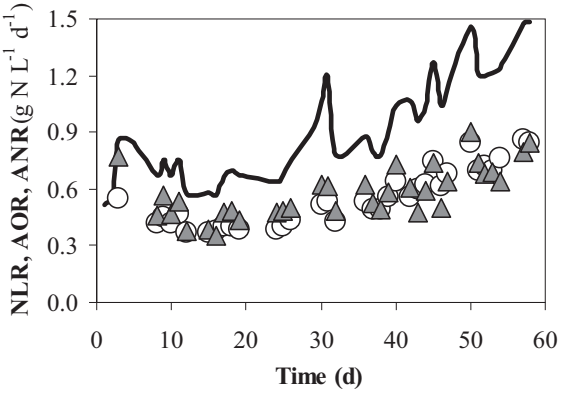

Figure 7.2. Nitrogen loading rate applied (—) Ammonia oxidation rate (○), nitrogen removal rate (▲).

The removal of total organic carbon accounted for 0.11 g TOC L^{-1} d^{-1}. Since this value is low and the main part of this carbon would be removed aerobically or it is expected to be recalcitrant, the amount of nitrogen removed by denitrifying heterotrophic bacteria can be neglected in comparison to the autotrophic nitrogen removal. For this reason, heterotrophic denitrification was not considered in the nitrite balance (Eq. 7.8) and the totality of the removed nitrogen was considered due to Anammox activity.

The biomass concentration inside the reactor remained almost constant at 5.7 g VSS L^{-1} during the 60 days of operation. The average diameter of the granules in the reactor was of 5 mm (Fig. 7.3). The value of the SVI_5 was 25 mL (g VSS)$^{-1}$ and the settling velocity of the granular sludge was of 110 m h^{-1}. The number of granules in the reactor was estimated as 2233 (Eq. 7.11) using a value of density of 29 g VSS $(L_{granule})^{-1}$ and the aforementioned biomass concentration.

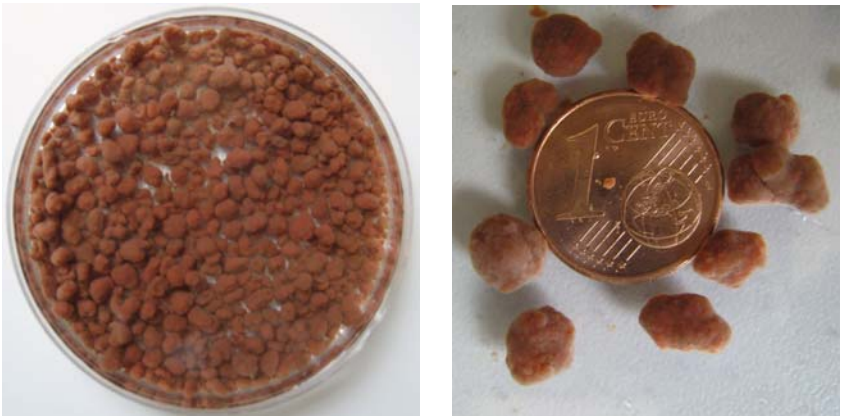

Figure 7.3. a) Photograph of several granules in a Petri plate b) Size comparison of some granules with a 1 cent of € (Diameter of the 1 cent of € = 1.6 cm).

The biomass concentration inside the reactor remained almost constant at 7.5 g VSS L^{-1} during the 60 days of operation. The average diameter and density of the granules were 5 mm and 36 g VSS $(L_{granule})^{-1}$, respectively. With these data, the number of granules was estimated using Eq. 7.11 giving the following result: 2230 granules. The value of the SVI was 25 mL $(g\ VSS)^{-1}$ and the settling velocity of the granular sludge was of 110 m h^{-1}.

7.4.2. Identification of bacteria populations by FISH

The stratification of the AOB and the Anammox bacteria in depth inside the granule can be observed in Fig. 7.4. In the outermost layer of the granule of 200 μm almost all the biomass was composed exclusively by *Nitrosomonas* spp. which gave positive signals with probes NEU653 and Nso190. Bacteria belonging to the genus *Nitrosomonas* spp. were still present until a depth of 600 μm but their proportion decreased along the depth (Fig. 7.5). *Nitrosomonas* spp. were determined as the main AOB community as expected due to conditions of ammonium excess (Schramm et al., 1998).

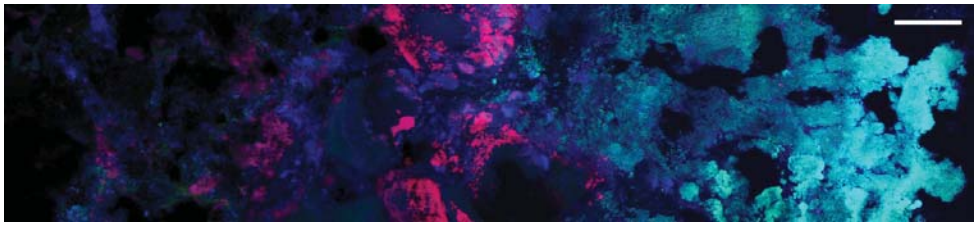

Figure 7.4. Image of a cryosectioned slice of a granule with a triple hybridization of FISH probes: NEU653 (light green, FLUOS); AMX820 (pink, Cy3) and EUBmix (blue, Cy5).The right part of the picture corresponds to the surface of the granule (the bar corresponds to 75 μm and the total width of the image corresponds to 1100 μm).

No significant fluorescence signal was detected with probe NIT3 specific for *Nitrobacter* spp. and neither with probe Ntspa712 specific for *Nitrospira* phylum. Therefore, no NOB activity was expected in the granules despite the high DO concentration in the liquid bulk.

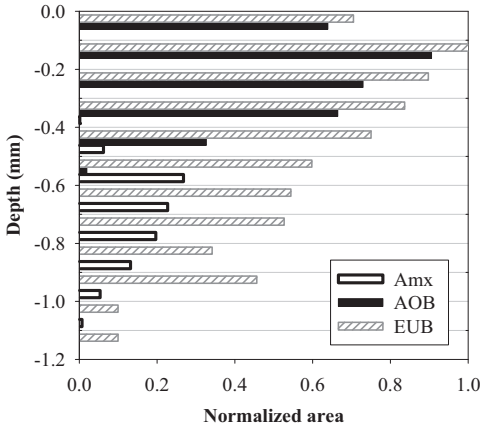

Figure 7.5. Depth distribution of AOB populations (hybridized with probe NEU653), Anammox bacteria (hybridized with probe Amx820) and all bacteria (hybridized with EUBmix) inside the granule. The value of depth equal to 0 mm corresponds to the granule surface.

Anammox bacteria were mainly located between 400 and 1000 μm of depth inside the granule where absence of dissolved oxygen is ensured. Bacteria belonging to the genus *Candidatus* Kuenenia stuttgartiensis were identified as the main Anammox bacteria in the reactor through positive results with probe Kst157. No positive results with probe Ban162 were obtained indicating absence of *Candidatus* Brocardia anammoxidans. Therefore, in the depths ranging from 400 μm to 600 μm AOB and Anammox bacteria coexist.

It is also interesting to point out that the area corresponding to EUBmix probe which represent all bacteria decreased along the granule, therefore, the activity of the granule is mainly located in the first 1000 μm which is in contact with the bulk liquid. Other bacteria (marked with EUBmix but not with NEU653 and Amx820) increased in proportion by going deeper inside the granule and they could correspond to those heterotrophs which possibly grew on soluble metabolic compounds (Kindaichi et al., 2004).

7.4.3. Microprofiles measurements

7.4.3.1. Oxygen microprofiles in the partial nitrification zone

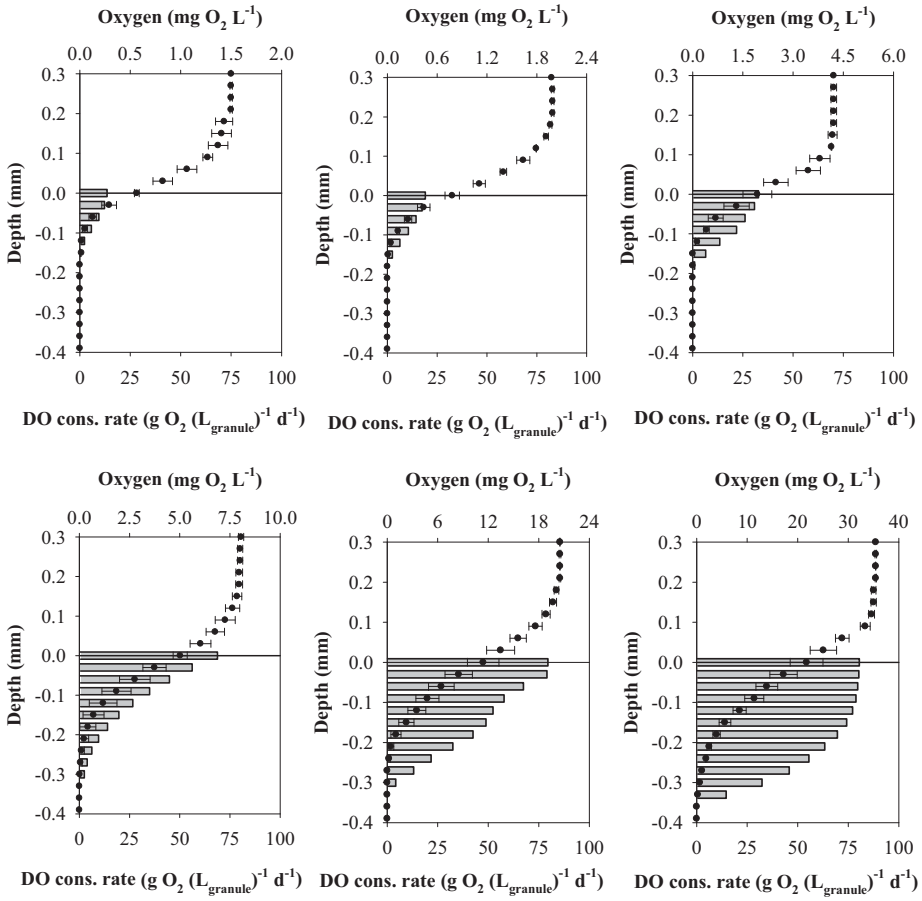

Figure 7.6. DO concentration profiles (●) and local consumption rates (■) under different DO concentrations in the bulk liquid (number of microprofiles (n): n=3 for all cases except for DO=8 mg O_2 L^{-1} where n=20).

In order to determine the oxygen consumption kinetics, microprofiles along the granule were performed varying the DO concentration in the bulk liquid in a wide range, from 1.5 to 35.2 mg O_2 L^{-1} (Fig. 7.6) with an initial ammonium concentration of 140 mg N L^{-1}.

The shape of the concentration profiles was similar for all profiles illustrating the various steps of oxygen transport and consumption: first the diffusion of DO through the external DBL and below that, the internal diffusion along with the biological reaction resulting in a curvature. The external transfer resistance was significant as a large decrease in DO concentration could be observed between bulk liquid and granule surface. This significant decrease in DO concentration within the DBL above highly active substrates have been reported previously by other authors (Jørgensen and Revsbech, 1985; de Beer et al., 1993; Rasmussen and Lewandowski, 1998; Wilen et al., 2004) and it highlighted the importance of the external mass transfer resistance, especially when working with granules as large as 5 mm. In the present study the width of the external DBL was around 100 - 120 μm.

Regarding the DO microprofiles inside the granules, it was observed that the oxygen penetration depth increased from 100 to 350 μm when increasing the DO in the bulk liquid from 1.5 to 35.2 mg O_2 L^{-1} (Fig. 4). The microprofiles revealed that even when the DO concentration in the bulk liquid was kept at 8 mg O_2 L^{-1} (close to 100% of air saturation), the maximal oxygen consumption rate was only attained in the outer part of the granule (corresponding to a depth of around 30 μm). Moreover, a fast decrease in the DO concentration with depth in the granule was registered in all the cases demonstrating that the oxygen mass transfer rate strongly limited the ammonia oxidation process. The high oxygen demand in the surface layer allowed an anoxic zone to be created in the internal part where the Anammox process could take place.

An estimation of the affinity constant of AOB was performed using the profiles obtained with the highest DO concentrations in the bulk liquid. The value obtained was 0.6 mg L^{-1}, which is in agreement with values previously published for AOB at 20 ºC (Wiesmann, 1994; Guisasola et al., 2005).

According to Harremoes and Henze (2002) the reaction rate as a function of the concentration of substrate outside a biofilm can be modelled by 3 types of kinetics: 1; 1/2 and 0 order. A kinetic order of 1 is a good approximation when the substrate concentrations in the bulk liquid is lower than $2 \cdot K_S$ (K_S is the half saturation constant for the substrate S), and a kinetic order of 0 is obtained when the biofilm is fully penetrated by the substrate (assuming a low K_m value). Half order kinetics represents the transition between 1 and 0 order and is caused by progressively deeper substrate penetration into the biofilm with increasing bulk concentration. In Fig. 7.7, the AOR is represented versus the square root of DO concentration at the surface of the granules. The AOR in the SBR was estimated using Eq. 7.12 considering the stoichiometry requirements of ammonium oxidation: 3.4 g O_2 (g NH_4^+-N)$^{-1}$. As it was expected according to Harremoes and Henze (2002), the tendency of the values is linear, i.e. 1/2 order kinetic, and even when working with pure oxygen in the bulk liquid, the granule was oxygen limited. Wilen et al. (2004) estimated that the DO required to obtain a 0 order kinetic was higher than 20 mg O_2 L^{-1} in aggregates with a diameter of 0.50−0.69 mm, and it is thus not surprising that the experiments with much larger aggregates exhibited half order kinetics These results illustrate the key role of the mass transfer limitations on the overall biomass activity in granular sludge.

The reactor was usually operated at DO concentrations of 6.6 mg O_2 L^{-1} meaning that the oxygen is available only in the first 200 μm of the granule. However when the DO concentration in the bulk liquid was increased up to pure DO saturation, progressively deeper layers were active which indicates that nitrifying bacteria might be able to remain alive in the absence of substrates and very quickly be activated as soon as

NH_4^+ and DO are provided. Such long term survival of nitrifying bacteria under ammonium- starved or anoxic conditions has previously been reported (Wilhelm et al. 1998, Freitag and Prosser 2003).

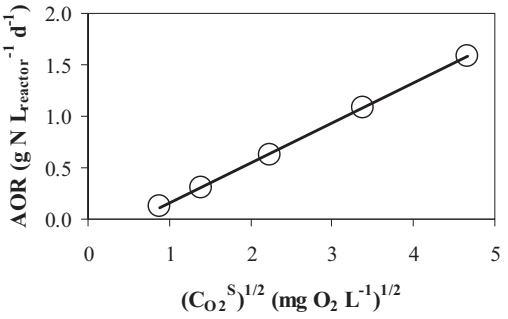

Figure 7.7. AOR estimation from oxygen microprofiles vs. square root of DO concentration at the surface of the granule (with trend line).

In Table 7.2, oxygen profiles along nitrifying granules/biofilms reported in literature are indicated. The different biofilms or granules analyzed came from different reactors with different hydrodynamics characteristics: rotating disk reactors (Okabe et al., 1999; Kindaichi et al., 2006); fluidized bed reactors (de Beer et al., 1993; Schramm et al., 1999); sequencing batch biofilm reactor (Gieseke et al., 2003) and granular SBR (Wilen et al., 2004; this study). The DBL thickness ranged between 91 and 140 μm and a considerable DO drop through the diffusive boundary layer was always observed which show the importance of the external mass transfer resistance. The nitrifying activity was mainly concentrated in the first 100 μm resulting in a steep gradient in DO concentration.

Table 7.2. Comparison of DO microprofiles in autotrophic nitrifying biofilms.

DO_{bulk} (mg O_2 L^{-1})	**$DO_{surface}$** (mg O_2 L^{-1})	**$DO_{100\mu m}$** (mg O_2 L^{-1})	**T** (ºC)	**Biofilm width** (μm)	**DBL** **δ_h** (μm)	**J_{O2}** (g O_2 m^{-2} d^{-1})	**Ref[d]**
8.0	2.9	0.2	30	1000-1250[c]	120	10.5[a]	[1]
7.4	4.5	1.3	30	500-1500[c]	100[a]	10.6[a]	[2]
8.0	2.6	0.2	25	200-500	100	5.9[b]	[3]
7.4	3.7	1.3	20	200	140	5.0[a]	[4]
6.1	3.0	0.6	20	350	100	5.2	[5]
8.0	4.8	1.4	20	2500[c]	108	5.1	T.S.
2.0	0.5	0.0	25-27	400	91[a]	2.8[b]	[6]
2.0	0.8	0.1	20	2500[c]	120	1.8	T.S.

[a] Values published by the authors in the reference. The value published by Kindaichi et al., (2006) was 1.1 g N m^{-2} d^{-1} for ammonium and it was converted to oxygen flux with the stoichiometric coefficient (4.57 g O_2 per g NH_4^+-N for complete nitrification).

[b] Values estimated considering an oxygen diffusivity at 25ºC of $1.9 \cdot 10^{-4}$ m^2 d^{-1} (Gieseke et al., 2003).

[c] Radius of the granules.

[d] [1] Schramm et al., (1999) Values corresponding to the port A of the fluidized bed reactor [2] de Beer et al., (1993) [3] Gieseke et al., (2003) [4] Kindaichi et al., (2006) [5] Okabe et al., (1999) [6] Wilen et al., (2004) Values corresponding to the upstream microprofile of the reference. T.S. = this study.

7.4.3.2. Nitrite microprofiles in the partial nitrification zone

NO_2^- microprofiles were determined under conditions of ammonium excess (initial NH_4^+ concentration in the bulk liquid of 140 mg N L^{-1}), starting with low concentration of nitrite in the bulk liquid and at different DO concentration. NO_2^- microprofiles corresponding to the three higher DO concentrations tested are represented in Fig. 7.8. Low NO_2^- concentrations in the bulk liquid were used in order to obtain a clearly visible nitrite peak in the nitrification zone allowing calculation of nitrification rate profiles. The NO_2^- concentration at the surface of the granules was significantly higher than the NO_2^- concentration in the bulk liquid.

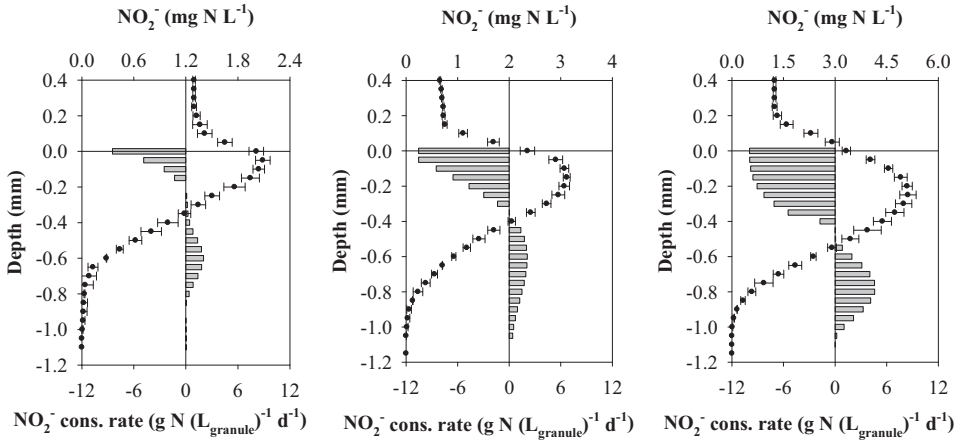

Figure 7.8. Nitrite profiles (●, with standard deviation, n=3) and local consumption rates (bar) under different O_2 concentrations in the bulk liquid: a) DO=8.0 mg O_2 L^{-1} b) DO=20.5 mg O_2 L^{-1} c) DO=35.2 mg O_2 L^{-1}. The NO_2^- concentration in the aqueous phase was kept at low values around 1 mg L^{-1}.

Two well defined zones could be differentiated (Fig. 7.9): 1) the nitrification zone, corresponding to the external layers in contact with the bulk liquid where nitrite was produced by AOB and 2) the anammox zone where NO_2^- was consumed together with NH_4^+ under anoxic conditions.

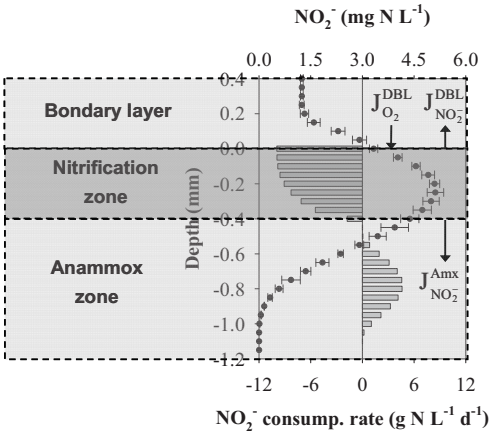

Figure 7.9. Representation of the fluxes of oxygen and nitrite in the granules.

From the microprofiles it is observed that the nitrite peak and the nitrite production rate became higher by increasing the DO concentration in the bulk liquid. Due to the gradient of concentrations produced in the nitrification zone, nitrite diffused to both bulk liquid and inner layers of the granule. These concentration gradients were caused by the nitrite generation in the nitrifying zone, its diffusional transfer to the bulk liquid during continuous operation, and the NO_2^- consumption in the inner layers by anammox bacteria. However, the steepness of the NO_2^- gradient in the DBL is an artefact caused by the low NO_2^- concentration in the bulk liquid, and in the reactor there should on the average be a considerably lower net flux to the liquid phase than suggested by our model example.

The zone of NO_2^- production obtained with the NO_2^- microsensor corresponds well with the one obtained using the DO microsensor. A lower level of details is obtained due to the higher discretization step (50 μm instead of 25 μm) but the Monod kinetic is also well reflected with higher activities in the external layers. In the same way that DO consumption, for the case of pure DO saturation, the 200 μm more external layers are working at maximal activity.

The consumption rates of oxygen were five times higher than the nitrite production rate. This ratio is superior to the stoichiometrically required (according to the stoichiometry of ammonia oxidation to nitrite, the ratio would be 3.4 g O_2 (g N)$^{-1}$). The different values can be attributed probably to a mismatch in the NO_2^- diffusivity. The NO_2^- diffusivity might be enhanced/decreased by the presence of anions and cations in the nitrification zone which could cause that the ratio diffusivity NO_2^-/diffusivity O_2 in the nitrification zone has a different value than the one in pure water. The fact that the nitrite peak is displaced to more internal layers when increasing the nitrifying activity may also corroborate the difference in the nitrite diffusivity or the higher Anammox activity. In all the cases the NO_2^- flux is higher to the bulk liquid, which could also mean that the nitrite diffusion outwards of the granule is easier than the diffusion inwards.

The fluxes of NO_2^- out of the nitrification zone, the total flux value and the flux to the core of the granule, are shown in Table 7.3. As it was expected, the fluxes of nitrite towards the bulk liquid and towards the Anammox zone increased when the O_2 concentration in the bulk liquid increased.

Table 7.3. DO and NO_2^- fluxes in and out of the nitrifying zone at different DO concentrations in the bulk liquid.

DO_{bulk} (mg O_2 L^{-1})	J_{O2}^{DBL} (g O_2 m^{-2} d^{-1})	$J_{NO2}^{Total} = J_{NO2}^{DBL} + J_{NO2}^{Amx}$ (g N m^{-2} d^{-1})	J_{NO2}^{Amx} (g N m^{-2} d^{-1})	J_{O2}^{DBL} $(J_{NO2}^{Total})^{-1}$ (g O_2 (g N)$^{-1}$)
35.2	20.2	2.7	1.0	7.5
20.5	11.9	2.3	0.7	5.2
8.1	5.1	1.3	0.4	4.0

7.4.3.3. Anammox zone

In order to obtain the maximal Anammox activity inside the granule, neither ammonium nor nitrite concentrations can limit. Therefore, high concentrations in the bulk liquid of ammonium (initial concentration of 140 mg NH_4^+-N L^{-1}) and nitrite (from 8.4 to 42.0 mg NO_2^--N L^{-1}) were applied while DO concentration in the bulk liquid was air saturation (8 mg O_2 L^{-1}). Nevertheless the CANON reactors are normally operated under nitrite limitation since it is necessary to keep the Anammox potential higher than the nitrification potential in order to avoid the occurrence of fatal nitrite build-up which is usually followed by irreversible nitrite inhibition of the Anammox biomass (Nielsen et al., 2005).

Even in those experiments performed with pure oxygen saturation, the NO_2^- concentration in the bulk liquid of 1.3 mg N L^{-1} was not sufficient to attain maximal Anammox activity since NO_2^- became zero in internal

layers where Anammox bacteria were still present. The strategy followed to estimate the maximal Anammox activity was to maintain the DO concentration at 8 mg O_2 L^{-1} (granular SBR conditions) and to increase the NO_2^- concentration in the bulk liquid. In the experiment carried out at a nitrite concentration in the bulk liquid of 9.1 mg N L^{-1} (Fig. 7.10), the nitrite concentration obtained in the inner core of the granule was 0.7 mg N L^{-1}. Since nitrite half saturation constant of Anammox bacteria was reported to be less than 0.1 mg N L^{-1} (Strous et al., 1999), maximal Anammox activity in the granule was ensured.

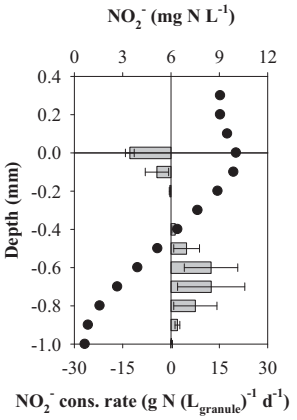

Figure 7.10. Nitrite concentration profile (●) and the average local consumption rate (■; bars indicate standard deviation: n=6).

From the nitrite profiles obtained in Fig. 7.8 and Fig. 7.10 it can be inferred that, by keeping the O_2 in the bulk liquid constant, the increase of the NO_2^- concentration in the bulk liquid increases the flux of nitrite to the Anammox zone until a maximum value of 1.0 g N m^{-2} d^{-1} is reached. This flux of nitrite to the anoxic layers of the granule is expected during the SBR operation since the mean nitrite concentration in the bulk liquid ranged between 14 and 42 mg N L^{-1}. Such high fluxes of nitrite to the anoxic layers of the granule are expected during the SBR operation since the mean nitrite concentration in the bulk liquid ranged between 12 and 42 mg N L^{-1}.

Kindaichi et al., (2007) obtained similar maximal nitrite removing capacity with a maximal volumetric rate of 5.0 g N $(L_{biofilm})^{-1}$ d^{-1} and a similar NO_2^- flux of 2.2 g N m^{-2} d^{-1} performing microprofiles in the biofilm of an anaerobic fixed bed column with the difference that they worked at 37 ºC. However in their case, the maximal Anammox reaction width was the upper 1300 μm of the biofilm, whereas in the present study, Anammox bacteria were located in the range of depths between 400 and 1000 μm.

Using the information of the NO_2^- microprofiles, an estimation of the nitrogen removal rate of the SBR using Eq. 12 was performed. With these data and taking into account the stoichiometry of the Anammox reaction, an ANR of 0.5 g N L^{-1} d^{-1} was estimated in the SBR. This value was close to the mean ANR value obtained in the reactor (Fig. 1). Our microscopic analysis of the granules was thus in agreement with macroscopic observations.

7.4.3.4. Causes of error

In order to better explain the obtained results, the different assumptions made during the performance of the experiments with the microsensors and the corresponding calculations have to be analyzed. As it was indicated the calculations were based on the assumption that the granules are described by flat surfaces,

whereas the granules were sphere shaped and thus this fact could influence the flow pattern. Besides, it was assumed that the granules were homogeneous in structure. It has been widely demonstrated that biofilms are heterogeneous and therefore diffusivities should be defined locally. A simple cut made to the granule (Fig. 7.11) corroborates the high heterogeneity of the aggregates.

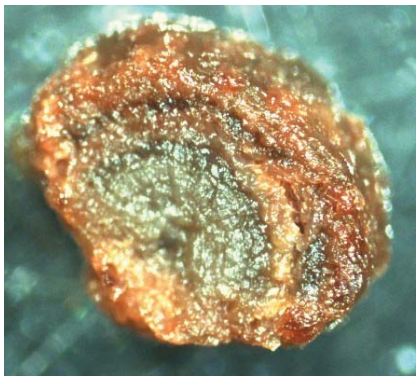

Figure 7.11. Photograph of a transversal of granule from the CANON reactor.

However, it was beyond the scope of this article to study in depth the heterogeneity of the bacteria populations and the approximations done are satisfactory to give important information on the processes carried out into the granule. Finally, the surface layer of the granule was somewhat fluffy, where a visual definition of "surface" within ± 50 μm was difficult. By performing several repetitions, the high values obtained for the standard deviations confirmed the high variability of the results, but, as it will be further explained, the approximations were satisfactory since the microscopic results predicted fair enough the macroscopic ones.

7.4.4. From microscale results to granular CANON operation

A successful strategy to start-up a CANON reactor is the promotion of the growth of the AOB population in the form of granular biomass. AOB produce nitrite and consume oxygen to provide anoxic conditions in the inner core of the granules. In this anoxic zone, ammonium (left from the AOB activity) and nitrite (from partial nitrification) have to be present in order to allow the growth of Anammox bacteria (Vázquez-Padín et al., 2009a,b). The activity of the external layer mainly composed by AOB should be thick enough to protect Anammox bacteria from the penetration of dissolved oxygen but its activity must be controlled to avoid inhibition of Anammox bacteria by high nitrite concentrations. This inhibition can occur when the diffusion rate of nitrite to the anoxic zone is higher than the nitrite consumption by Anammox bacteria. In this sense, data obtained with the application of microelectrodes to granules performing the CANON process allows knowing both optimal and safe conditions to operate the CANON system, in terms of ammonia and nitrite concentrations in the bulk liquid. At this point a control strategy could be defined to regulate the concentration of both compounds by using on-line ammonia and nitrite analyzers. The variables of control would be the DO concentration and the HRT value using the air flow rate and the inlet flow rate as actuation elements.

Different scenarios for the different ranges of ammonium and nitrite concentrations are possible (Fig. 7.12a). Low NH_4^+ and NO_2^- concentrations would limit the activities of AOB and/or Anammox bacteria. Assuming that the diffusion coefficients of NH_4^+ and O_2 are barely similar, AOB would be limited by ammonia when the ratio DO/NH_4^+ in the bulk liquid is lower than 3.4 g O_2 (g $N)^{-1}$. The limitation of AOB by ammonia would expose the reactor to a risk of failure since the granules would be fully penetrated by oxygen which

would temporally inhibit Anammox bacteria and enhance the undesired growth of NOB (Sliekers et al., 2005). On the other hand, high NH_4^+ and/or NO_2^- concentrations have the following disadvantages: both substrates can inhibit AOB and Anammox bacteria with a consequent detriment of the produced effluent quality. Moreover, maintaining concentrations of NO_2^- higher than the optimal ones involves higher costs of aeration.

The control strategy corresponding to the different defined zones to restore the optimal conditions will be: a) in case of nitrite concentration out of the optimal range to act over the DO concentration (increasing or decreasing its value); b) whereas to optimize the NH_4^+ concentration the value of the HRT will be modified as the control parameter; and in the case where NH_4^+ and NO_2^- concentrations were not in the optimal range, both, HRT value and DO concentration would be changed according to Fig. 7.12b.

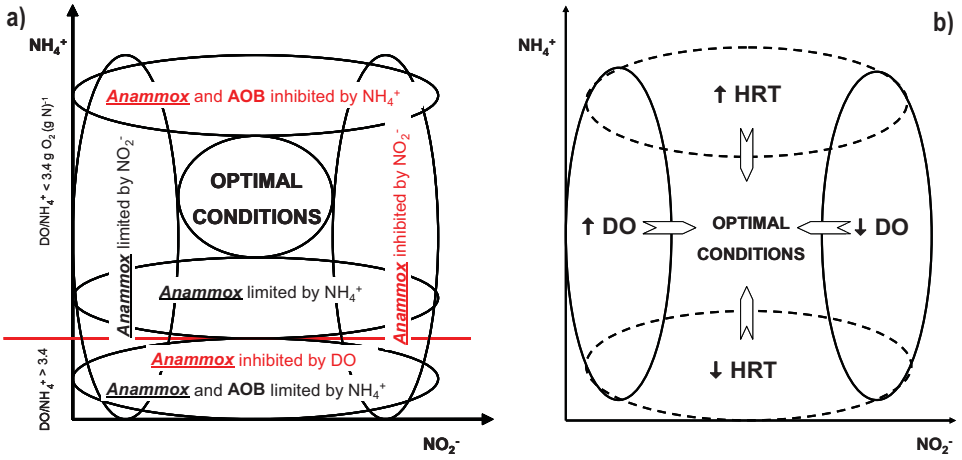

Figure 7.12. a) Zones defining the different operational conditions in a CANON reactor fed with different NH_4^+ and NO_2^- concentrations in the bulk liquid. b) Control strategy to return the CANON reactor to optimal conditions depending on NH_4^+ and NO_2^- concentrations in the bulk liquid.

Making use of the previous qualitative analysis the optimal values for NH_4^+ and NO_2^- concentrations can be defined for a specific case. These parameters will be determined specifically for the biomass present in each CANON reactor. An example would be given for the granules operated in the CANON SBR from this work. As it was obtained from the microprofiles measured into the granules, the nitrite concentration has to be at least 9 mg N L^{-1} (Fig. 7) to ensure maximal activity of Anammox bacteria in the anoxic core of the granules. Ammonium microprofiles were not performed in this study, however, similar behaviour is predictable and a minimum ammonium concentration in the bulk liquid will be required to ensure maximal Anammox activity. Taking into account that the reactor was operated at a DO concentration close to 8.0 mg O_2 L^{-1} in the bulk liquid, the DO concentration at the surface of the granule would be 4.8 mg O_2 L^{-1} (Table 2). Therefore the NH_4^+ consumed in the nitrification zone assuming that only AOB were present in this zone and that the diffusivity of O_2 and NH_4^+ are identical (stoichiometry requirements: 3.4 g O_2 (g NH_4^+-N)$^{-1}$) will be 4.8/3.4=1.4 mg N L^{-1} of NH_4^+. In the Anammox zone, 9 mg N L^{-1} of nitrite would be removed and therefore, according to the Anammox stoichiometry and again assuming identical diffusion coefficients, 7 mg N L^{-1} of NH_4^+ would also be consumed. Therefore, to avoid NH_4^+ limitation its concentration in the bulk liquid has to be larger than 8.4 mg N L^{-1} while the NO_2^- concentration has to be larger than 9 mg N L^{-1}. It should be stressed that the calculations above are crude as the diffusion coefficients are assumed to be identical for O_2, NH_4^+, and NO_2^-, which is not the case.

There must, however, be equilibrium between diffusional transport of negative and positive ions between the aggregates and the bulk liquid, and this may as already mentioned result in diffusivities that are different from the self-diffusion coefficients (Reimer and Harremoes 1978) so that the actual diffusion coefficients are even more different than the about ±10% suggested by literature (Li and Gregory, 1974; Picioreanu et al. 1997).

The fact that a minimal concentration of both ammonium and nitrite are necessary in the bulk liquid to ensure maximal activity of anammox bacteria can represent a drawback if the effluent of the CANON reactor has to be released to a natural media but has not a significant impact if the effluent is returned to the head of the WWTP.

7.5. Conclusions

● Ammonia oxidation to nitrite is highly dependent on the oxygen mass transfer. Oxygen limitation regulates the amount of nitrite produced and as a consequence influences the Anammox activity. Nitrite microprofiles revealed that a minimum nitrite concentration around 9 mg N L^{-1} is necessary to ensure that Anammox biomass is working at maximal activity.

● Oxygen and nitrite microprofiles determined by application of microsensors correlated to the distributions of AOB and Anammox bacteria inside the granules.

● The bacterial activities estimated from microscale analysis (0.5 g N L^{-1} d^{-1}) gave similar results to those obtained from macroscopic measurements in the granular SBR.

● An accurate regulation of the dissolved oxygen concentration in the bulk liquid and the HRT value are crucial to control the nitrogen removal process in a CANON system avoiding either limitations or inhibitory phenomena.

7.6. References

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Chapter 8

Modelling aerobic granular SBR at variable COD/N ratios including accurate description of total solids concentration[1]

Summary

The operation of a sequencing batch reactor (SBR) with aerobic granular biomass was successfully simulated using a one-dimensional biofilm model. The biological processes considered were described based on the Activated Sludge Model (ASM) platform with two main modifications: (i) simultaneous growth and storage of organic substrates by heterotrophic bacteria; and (ii) inclusion of nitrite as intermediate compound in the nitrification and denitrification processes. Three different operational conditions were evaluated, characterized by different chemical oxygen demand to nitrogen (COD/N) ratios in the influent of: 0, 1.25 and 5.5 g g^{-1}, representing a purely autotrophic media and two heterotrophic media, respectively. An accurate description of the experimental concentrations of COD, ammonium, nitrite, nitrate, dissolved oxygen (DO) and alkalinity along the cycles was obtained. Total solids concentration inside the reactor (5.0, 2.0 and 1.0 g VSS L^{-1} for COD/N ratio of 5.5, 1.25 and 0 g g^{-1}, respectively) and biofilm density (23 g $(L_{granule})^{-1}$) were correctly described with the model. To obtain an accurate description of both solids concentration and biofilm density different densities were defined for the particulate compounds and a porosity profile along the granule was imposed. Oxygen penetration depths obtained with the model were 0.35, 0.30 and 0.12 mm for COD/N ratio of 5.5, 1.25 and 0 g g^{-1}, respectively. The values were in agreement with those used in the description of the porosity profiles.

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

8.1. Introduction

Activated sludge systems are one of the most common technologies used for biological wastewater treatment. However, in this kind of systems, the treated loading rates are limited by the biomass retention capacity due to the poor settling properties of the sludge. Accumulation of large biomass concentrations inside the systems requires the installation of big settlers with large space requirements. Furthermore the activated sludge systems frequently experience problems of biomass bulking with the consequent solids washout and commonly the loss of the nitrification capacity.

To overcome the drawbacks associated with activated sludge systems, in the 1990s, the use of biofilm systems for aerobic processes was promoted. But the main disadvantage of these systems is the relatively high investment costs associated to the necessity of using support materials for the biomass attachment. In order to get the benefits of biofilm systems, but without the use of carrier materials, in recent years, research showed that it is possible to grow aerobic granular sludge in either continuous or batch-wise operated systems (Beun et al., 1999; Campos et al., 2000). Compared to conventional activated sludge flocs, aerobic granules present regular, dense, and strong physical structure, good settling ability, allow high biomass retention in the systems, and possess the ability to withstand shock loads and to maintain a good efficiency of nitrogen removal (Yang et al., 2003).

The first studies regarding aerobic granular sludge systems were performed in the 1990s in continuous operated systems (Mishima and Nakamura, 1991). In recent years an increasing number and diversity of studies reflecting research on aerobic granular sludge were developed and showed that it is possible to grow aerobic granules in Sequencing Batch Reactors (SBRs) treating different kinds of wastewater. These systems have been used for organic carbon removal (Beun et al., 1999; Dangcong et al., 1999; Etterer and Wilderer, 2001; Tay et al., 2002; Sun et al., 2006) and simultaneously for nutrient (nitrogen and phosphorus) removal (Beun et al., 2001; Lin et al., 2003; de Kreuk et al., 2005; Yang et al., 2005; Wang et al., 2007).

Due to the interest in increasing the knowledge about the operation of the biofilm and granular systems different mathematical models have been developed. Mathematical modelling of biofilms has gained increasing interest in the last decades and a detailed overview has been published by Wanner et al., (2006) covering a wide range of biofilm models of different complexity applied to different types of benchmark problems. Using the experience in biofilm modelling one dimensional (1-D) models have been adapted to describe nutrient removal in aerobic granular SBR (GSBR). Su and Yu (2006a, b) used the ASM1 (Henze et al., 2000) describing the maximum specific growth rates of heterotrophic and autotrophic bacteria with exponential functions, whose parameters were adjusted to describe the experimental nutrient removal observed in the reactor. Lübken et al., (2005) used the ASM3 (Henze et al., 2000) modifying two model parameters through a calibration procedure: (i) the aerobic endogenous respiration coefficient and (ii) the half-saturation coefficient for oxygen, both for the heterotrophic biomass. Nevertheless, models developed by these authors did not solve the reaction-diffusion partial differential equations.

In this sense, other authors developed one-dimensional (1-D) biofilm models taken into account these reaction-diffusion partial differential equations. Beun et al., (2001) simulated the operation of a GSBR reactor with biomass grown as granules of an average diameter of 2.5 10^{-3} m and the sludge retention time (SRT) of 50 days. They showed that nitrification, denitrification and COD removal can occur simultaneously in a GSBR. Later, de Kreuk et al., (2007) introduced the phosphorous removal in addition to the COD and N removal and studied how the removal of nutrients was affected by several parameters like the temperature of operation and

the diameter of the granules. Ni et al., (2008) utilized a modification of the ASM3 model to describe the simultaneous autotrophic and heterotrophic growth in aerobic granular SBR and demonstrated that the influent ammonia concentration determined the composition and distribution of heterotrophic and autotrophic biomass in an aerobic granular SBR.

Recently, more complex modelling approaches have also been presented to describe nutrient removal in GSBR. The development of multidimensional (2-D or 3-D) models offers more potential to predict local compositions of particulate and dissolved variables. 2-D and 3-D biofilm models could be divided in two classes according to the way of describing the biomass: as discrete individual particles or as a continuum body. Biofilm models as discrete particles are the most suitable ones for extrapolation to granular biomass. Individual-based models (IbM) have been used to describe both the steady state conversions of this reactor type as well as its dynamic behaviour (Xavier et al., 2007). The results of this research showed that in GSBR systems the nitrogen removal occurs mostly via alternating nitrification/denitrification rather than simultaneous nitrification/denitrification.

The efforts carried out in the development of models were focused mainly on describing substrates (organic matter, nitrogen, phosphorous) removal but did not provide realistic information about biomass concentration and its properties (density and number of granules). To this effect the porosity depth profile in the granule should be considered since it affects the conversion, biofilm density and total biomass amount in the system (Horn and Hempel, 1997; Lewandowski, 2000; Laspidou and Rittmann, 2004). The porosity profile is linked to the shear stress in the reactor, the biomass inactivation and death, the consolidation of inner parts of the biofilm, the growth of anaerobic cells in the free spaces of inner biofilm layers, etc. (Laspidou and Rittmann, 2004). Biofilm porosity has been found to be heterogeneous, decreasing with biofilm depth (Zhang and Bishop, 1994; Tay et al., 2003). In fact microscopic observations indicated that aerobic granules comprise two quite different parts: an active shell with high porosity and an inner core with low water content and reduced activity (Tay et al., 2003).

8.2. Objectives

● The aim of the present work is to develop a 1-D model to describe the operation of an aerobic granular SBR treating an influent characterized by different COD/N ratios. Results of the simulations will be directly compared to the experimental results. In addition, special attention has been paid to the description of the total solids concentration in the reactor and the density of the granules for each studied condition.

8.3. Materials and methods

8.3.1. Granular Sequencing Batch Reactor (GSBR) operation

The experimental results were obtained in a laboratory scale SBR with a working volume of 1.5 L. Dimensions of the reactor were: height of 46.5 cm and inner diameter of 8.5 cm, the height to diameter ratio being 5.5. The exchange volume was fixed at 50%. Oxygen was supplied from the bottom of the reactor by using air spargers. A set of two peristaltic pumps was used to introduce the feeding solution (on top of the reactor) and to discharge the effluent (at medium height in the column reactor). A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations of the pumps and valves, and regulated the different periods of the operational cycle. The SBR was operated in cycles of 3 hours distributed as follows: 3 minutes of feeding, 171 minutes of aeration, 1 minute of settling and 5 minutes of withdrawal. The hydraulic

retention time (HRT) was fixed at 6 hours (a detailed description of the reactor operation is given in Mosquera-Corral et al., 2005).

The operational strategy consisted of the stepwise decrease of the COD/N ratio of the synthetic feed supplied to the reactor in order to obtain nitrifying granules (Table 8.1). $CH_3COONa \cdot 3H_2O$; NH_4Cl and $NaHCO_3$ were used as source of organic matter (COD), ammonium (NH_4^+) and alkalinity (Alk), respectively. Other compounds were also added to the feeding with the following composition in g L^{-1}: 0.092 K_2HPO_4; 0.036 KH_2PO_4; 0.049 $MgSO_4$; 0.019 KCl and 0.5 mL L^{-1} of a trace solution. The composition of the trace solution was in g L^{-1}: 1.50 $FeCl_3 \cdot 6\ H_2O$, 0.15 H_3BO_3, 0.15 $CoCl_2 \cdot 6\ H_2O$, 0.12 $MnCl_2 \cdot 4\ H_2O$, 0.12 $ZnSO_4 \cdot 7\ H_2O$, 0.06 $NaMoO_4 \cdot 2\ H_2O$, 0.03 $CuSO_4 \cdot 5\ H_2O$ and 0.03 KI.

Table 8.1. Operational conditions in the studied experimental periods: Concentrations of organic matter (C_S), ammonium (C_{NH4}) and alkalinity (C_{Alk}) in the influent of the reactor and corresponding COD/N ratio, Organic Loading Rate (OLR) and Nitrogen Loading Rate (NLR).

COD/N (g g^{-1})	C_S (g COD L^{-1})	C_{NH4} (g N L^{-1})	C_{Alk} (g IC L^{-1})	OLR (g COD L^{-1} d^{-1})	NLR (g N L^{-1} d^{-1})
5.50	0.55 ± 0.03	0.10 ± 0.01	0.00 ± 0.00	2.20	0.4
1.25	0.06 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.24	0.2
0.00	0.00 ± 0.00	0.10 ± 0.01	0.17 ± 0.01	0.00	0.4

The reactor was operated at room temperature 24 ± 1 ºC, the pH was not controlled and ranged from 7 to 8 whereas the DO concentration varied in the range 5—8 g O_2 L^{-1} in the bulk liquid with an air flow rate of 1.7 ± 0.2 L min^{-1}. The sludge retention time (SRT) ranged from 15 to 35 d. Granular biomass used in this work was previously developed in the same system treating wastewater from a dairy industry (Arrojo et al., 2004).

8.3.2. Analytical methods

The values of pH, DO, NH_4^+ and volatile suspended solids (VSS) concentrations were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Nitrite and nitrate concentrations were determined by capillary electrophoresis (Vilas-Cruz et al., 1994). Concentrations of total organic carbon (TOC) and inorganic carbon (IC) were measured with a Shimadzu analyser (TOC-5000). Density of the granules (g VSS $(L_{granules})^{-1}$) was measured using the dextran blue method described by Beun et al., (2002).

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) combined with a digital camera (Coolsnap, Roper Sicientific Photometrics). Digital image analysis was carried out using Image ProPlus software. Further information about the analytical methods is provided in Chapter 2.

8.4. Model development

AQUASIM (Reichert, 1998) was the chosen software for the implementation of the developed model. AQUASIM is a widely used program in the environmental research field for modelling purposes. On the basis of the models already published, specific assumptions were utilized to obtain accurate simulations of the concentrations of the different compounds in the bulk liquid as well as the biomass concentrations obtained at steady state under different COD/N ratios.

The simulations were run until less than 5% of variation in the biomass concentrations for the different species at any point of the granule depth were attained. This meant a simulation of a period of operation of about 100 days. Each simulation lasted up to 28 h of computing time on a 2.4 GHz microprocessor.

8.4.1. Definition of the SBR operation

The mathematical description of the discontinuous operation of the SBR consisted of linking a biofilm compartment to a completely mixed compartment with a high recirculation flow rate (Q_{rec} = 144 m^3 d^{-1}), in order to ensure the same bulk liquid concentration in both compartments (Beun et al., 2001; de Kreuk et al., 2005). The volume of the biofilm compartment was fixed at 0.65 L corresponding to the granules and the bulk liquid volume. The completely mixed compartment contained the remaining liquid and had a variable volume being its maximum value of 0.85 L which was kept constant during the aeration phase. This volume decreased to 0.1 L during the withdrawal phase and increased again to its maximal value during the feeding period.

8.4.2. Biological processes

The developed dynamic 1-D biofilm model included six soluble compounds: oxygen (C_{O2}), ammonium (C_{NH4}), nitrite (C_{NO2}), nitrate (C_{NO3}), alkalinity (C_{Alk}) (expressed in terms of inorganic carbon) and readily biodegradable organic substrate (S_S); and five types of particulate compounds: ammonia-oxidizing bacteria (X_A), nitrite-oxidizing bacteria (X_N), heterotrophic bacteria (X_H), storage products (X_{STO}) and inert particulate organic material (X_I). The biological processes introduced in the model were defined using the ASM modelling platform (Henze et al., 2000).

However, the ASM models (ASM1, ASM2, ASM2d and ASM3) present some limitations to describe the performance of an aerobic GSBR. In order to improve the description, two modifications were performed. The first one was the introduction of the simultaneous growth and storage of organic substrates by heterotrophic bacteria. This modification was already proposed by other authors (Krishna and Van Loosdrecht, 1999; Sin et al., 2005) for systems where feast and famine operating conditions are imposed repeatedly (e.g. GSBR). In this kind of systems, simultaneous aerobic/anoxic storage and growth on organic substrates by heterotrophs occur under feast conditions. The second modification was the inclusion of nitrite as intermediate compound in the nitrification and denitrification processes due to its importance in systems where nitrite accumulates (Jubany et al., 2008; Sin et al., 2008). Nitrification was defined as a two-step process with a first oxidation of ammonium to nitrite by ammonia-oxidizing bacteria (AOB) and a subsequent oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). Denitrification was described as two different processes, both processes carried out by heterotrophs under anoxic conditions and producing nitrogen gas from nitrite and nitrate, respectively.

Stoichiometric and kinetic parameter values together with the rate expressions are presented in appendixes A and B. All the kinetic parameters were taken from the literature. A temperature correction was applied through Eq. 8.1 for the kinetic parameters corresponding to heterotrophic biomass (i.e., k_{STO}, $\mu_{H,max}$, b_H and b_{STO}) as proposed by (Henze et al., 2000).

$$k\,(T) = k\,(20\,^{o}C)\cdot e^{0.07\,(T-20\,^{o}C)} \qquad (8.1)$$

where k is any of the kinetic parameters mentioned and T is the temperature (ºC).

The values corresponding to the kinetic parameters of autotrophic bacteria (i.e. $\mu_{A,max}$, $\mu_{N,max}$, b_A and b_N) were calculated with the equations proposed by Jubany et al., (2008) (Eq. 8.2-8.5) considering a temperature of 24 ºC and a mean pH of 7.5.

$$\mu_{A,max}\ (pH,T) = \frac{1.28\cdot10^{12}\cdot e^{\frac{-8183}{273+T}}}{1+\frac{2.05\cdot10^{-9}}{10^{-pH}}+\frac{10^{-pH}}{1.66\cdot10^{-7}}} \quad (8.2)$$

$$\mu_{N,max}\ (pH,T) = \frac{6.69\cdot10^{7}\cdot e^{\frac{-5295}{273+T}}}{1+\frac{2.05\cdot10^{-9}}{10^{-pH}}+\frac{10^{-pH}}{1.66\cdot10^{-7}}} \quad (8.3)$$

$$b_A\ (T) = 1.65\cdot10^{11}\cdot e^{\frac{-8183}{273+T}} \quad (8.4)$$

$$b_N\ (T) = 8.62\cdot10^{6}\cdot e^{\frac{-5295}{273+T}} \quad (8.5)$$

In Appendix C, the stoichiometric matrix of soluble and particulate compounds is represented in the format proposed by Henze et al., 2000. This format is a simplified way to corroborate the mass balances of COD and N. The balances are similar to those published by Kaelin et al., (2009) since they extended the ASM3 to incorporate the tow-step nitrification and denitrification processes. The main difference of the present model relies on the alkalinity balance since in the present study the organic substrate is the acetate ion which has a negative charge whereas in the ASM models the organic carbon source is considered as a compound without charge. In order to meet the electrochemical balance, when introducing an acetate ion into the cell, a negative charge will be produced (in the case of working in water, a hydroxyl ion will be released). Taking this into account the electrochemical balance was met and shown in the balances presented in Appendixes C, D and E. Appendix D is the numerical representation of the Appendix C and in the Appendix E the molar stoichiometrical equations are also indicated. In Table 8.1 and in Appendix D, the alkalinity is represented as IC since the bicarbonate ion was the main buffer system of the synthetic wastewater to keep the pH value in the range between 7 and 8.

8.4.2.1. Composition of particulate compounds

The stoichiometry of the biomass to describe X_H, X_A and X_N was defined according to Reichert et al., (2001) who expressed the mass fractions of the biomass as: 0.520 g C g $(X)^{-1}$; 0.080 g H $(X)^{-1}$; 0.250 g O $(X)^{-1}$ and 0.120 g N $(X)^{-1}$. The molar composition obtained from these mass fractions is as follows: $C_{5.06}H_{9.33}O_{1.82}N$ which is slightly different to the typically assumed composition of the biomass: $C_5H_7O_2N$. The composition of X_I was also estimated according to Reichert et al., (2001) but introducing the modifications performed during the calibration of the ASM3 (Koch et al., 2000), the mass fractions of the X_I are defined as: 0.610 g C g $(X_I)^{-1}$; 0.070 g H $(X_I)^{-1}$; 0.280 g O $(X_I)^{-1}$ and 0.075 g N $(X_I)^{-1}$. The molar composition of X_I obtained is as follows: $C_{4.74}H_{6.53}O_{1.63}N_{0.5}$. Finally, the last particulate compound, the storage products (X_{STO}), was defined with the formula C_2H_3O which corresponds to the chemical composition of poly-hydroxy-butyrate (Henze et al., 2000). The theoretical oxygen demand of the different particulate compounds was calculated using the data of the Table 8.2.

Table 8.2. Theoretical oxygen demand of the different elements involved in the model (Henze et al., 2000) and calculations of the ThOD of the particulate compounds.

Element		Equivalent ThOD
Carbon	C	+ 32 g ThOD (mol C)$^{-1}$
Hydrogen	H	+ 8 g ThOD (mol C)$^{-1}$
Oxygen	O	- 16 g ThOD (mol C)$^{-1}$
Nitrogen	N	- 24 g ThOD (mol C)$^{-1}$
Negative charge	-	+ 8 g ThOD (mol C)$^{-1}$
Positive charge	+	- 8 g ThOD (mol C)$^{-1}$

Biomass		Equivalent ThOD
$C_{5.06}H_{9.33}O_{1.82}N$	X_H, X_A, X_N	1.62 g ThOD (g VSS)$^{-1}$
$C_{4.74}H_{6.53}O_{1.63}N_{0.5}$	X_I	1.72 g ThOD (g VSS)$^{-1}$
C_2H_3O	X_{STO}	1.67 g ThOD (g VSS)$^{-1}$

8.4.3. Oxygen transfer

Gas-liquid oxygen transfer was considered in the model. An experimental estimation of the oxygen gas-liquid transfer coefficient (k_La) was carried out by means of the dynamic method described in chapter 2.

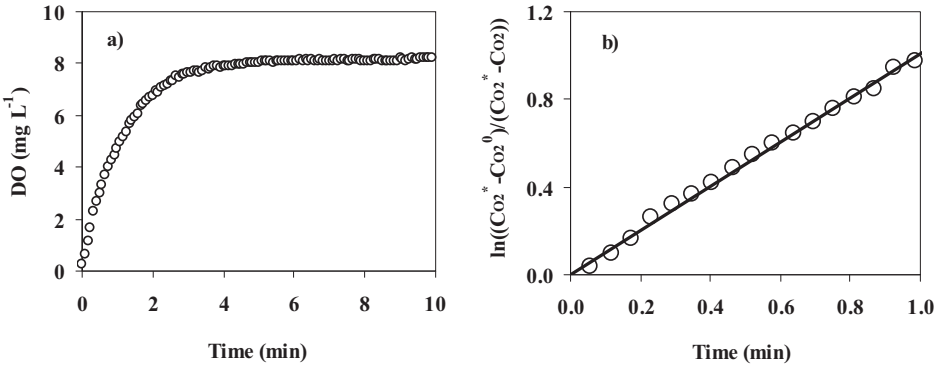

Figure 8.1. a) DO concentration in the reactor after aeration re-establishment b) Determination of the k_La coefficient.

The k_La coefficient was estimated at an air flow rate of 1.7 L min^{-1} and a temperature of 24 ºC, its value was 0.7 min^{-1} (or 1008 d^{-1}) with a regression coefficient equal to 0.998.

8.4.4. Granules description

Spherical coordinates were used to correctly describe the granule geometry. For each COD/N ratio applied (as described in Table 8.1) a mean value for the granule size was input to the model from experimental determinations. A volume-weighted average radius was experimentally determined (see Eq. 8.6) for each COD/N ratio tested (see Table 8.3).

$$R_m = \sqrt[3]{\frac{\sum_{i=1}^{n} R_i^{\,3}}{n}} \tag{8.6}$$

where R_m the weighted-mean radius (dm) of the granules and R_i the measured radius (dm) of each granule of a sample containing n granules.

The total number of granules in the reactor (n_T) to be used in the simulations was obtained from experimental data for each simulated period by dividing the total volume of granules (V_X) by the volume of a single granule (Eq. 8.7-8.8 and Table 8.3) (Beun et al., 2001).

$$n_T = \frac{V_X}{\frac{4}{3}\pi R_m^{\,3}} \tag{8.7}$$

$$V_X = \frac{V_R \cdot X_{total}^{exp}}{\rho_{granule}^{exp}} \tag{8.8}$$

being V_R the total reactor volume (L), X_{total}^{exp} the total solids concentration in the reactor (g VSS L^{-1}), $\rho_{granule}^{exp}$ the experimental density of the granules (g VSS $(L_{granule})^{-1}$). The experimental density did not differ significantly

between the different COD/N ratios studied. In the three operational stages the average value of the density was 23 ± 10 g VSS $(L_{granule})^{-1}$. The number of particles (n_T) used in each simulation is presented in Table 8.3.

Table 8.3. Experimental values of total solids concentration and radius of the granules, estimated volume of granules and number of granules for each COD/N ratio tested.

COD/N (g g^{-1})	X_{total}^{exp} (g VSS L^{-1})	**Average radius** R_m (mm)	**Volume of granules,** V_X (L) (from Eq. 8.8)	**Number of granules** (n_T) (from Eq. 8.7)
5.50	5.0 ± 1.5	2.0	0.325	9694
1.25	2.0 ± 0.5	2.0	0.130	3889
0.00	1.0 ± 0.4	1.3	0.065	6943

As it is shown in Table 8.2, the average diameter did not vary by using the COD/N ratios of 5.50 or 1.25 g g^{-1}, whereas it decreased by working with a COD/N ratio of 0 g g^{-1}.

8.4.5. Modelling the total solids concentration and density of the granules

Data corresponding to the concentration of each solid fraction for each discretization point (i) was used to obtain the total solids concentration as VSS in the reactor once each steady state was reached. X_{total}^{model} will be given by the sum of the total solids contained in one granule multiplied by the number of granules inside the reactor (Eq. 8.9).

$$X_{total}^{model} = \frac{n_T}{V_R} \cdot \sum_{i=2}^{N} \frac{\left(\frac{X_H^i}{1.62} + \frac{X_{STO}^i}{1.67} + \frac{X_A^i}{1.62} + \frac{X_N^i}{1.62} + \frac{X_I^i}{1.72}\right) + \left(\frac{X_H^{i-1}}{1.62} + \frac{X_{STO}^{i-1}}{1.67} + \frac{X_A^{i-1}}{1.62} + \frac{X_N^{i-1}}{1.62} + \frac{X_I^{i-1}}{1.72}\right)}{2} \cdot \frac{4}{3} \cdot \pi \cdot \left(R_i^3 - R_{i-1}^3\right) \quad (8.9)$$

being X_H^i, X_{STO}^i X_A^i, X_N^i, X_I^i, the concentrations of heterotrophs, storage compounds, AOB, NOB and inert particulate organic material respectively (g COD L^{-1}), R_i the radius of the granule in each discretization point (dm) and N the number of discretization points in the granule depth (i.e., number of grid points in AQUASIM). The conversion factor from COD to VSS for each particulate compound was established as defined in Table 8.2. The solids concentrations of each bacterial population in the granule can also be calculated using the Eq. 8.10, but considering only the solids concentration of the desired fraction.

The density of the granules estimated with the model ($\rho_{granules}^{model}$) was obtained by dividing X_{total}^{model} by the volume occupied by the granules present in the reactor (Eq. 8.10).

$$\rho_{granule}^{model} = \frac{X_{total}^{model} \cdot V_R}{n_T \cdot \frac{4}{3} \cdot \pi \cdot R_m^3} \quad (8.10)$$

8.4.6. Porosity of granules

As previously discussed in the introduction, the porosity of the granules is known to be heterogeneous along the granule depth (Tay et al., 2003), given that a decrease of the water content is found in deeper layers either of aerobic granules, or in biofilm systems (Zhang and Bishop, 1994; Lewandowski, 2000; Tay et al., 2003). Given that one of the main goals of the present contribution is to simulate accurately the biomass inside the reactor, a depth profile of porosity was selected and specifically developed.

The porosity of the deepest layer of the granule (i.e., the centre) was assumed to be 0.6, whereas for the external layer a value of 0.8 was imposed, based on the values published elsewhere (Zhang and Bishop, 1994; Lewandowski, 2000). A linear function was used to represent the variation of porosity along the inner (anoxic) zone of the granule (Eq. 8.11), whereas the porosity was considered constant through the outer (aerobic) shell (Eq. 8.12).

$$\varepsilon(r) = 0.6 + r\,\frac{0.2}{R_m - \delta} \qquad \text{if } 0 < r < R_m\text{-}\delta \tag{8.11}$$

$$\varepsilon(r) = 0.8 \qquad \text{if } R_m\text{-}\delta < r < R_m \tag{8.12}$$

where r is the radius (mm) at which the porosity (ε) is calculated, R_m the radius of the granule (mm) and δ is the oxygen penetration depth (mm).

The width of the external shell (δ), usually penetrated by oxygen, was roughly estimated by means of a basic calculation of the oxygen penetration depth. According to (Beun et al., 1999), an approximation of the oxygen penetration depth can be calculated assuming steady state conditions, spherical particles, zero order kinetics and no external mass transport resistance using Eq. 8.13. The penetration depth obtained was 0.25; 0.49 and 0.59 mm for COD/N ratios of 0; 1.25 and 5.5 g g^{-1} respectively.

$$\delta = \sqrt{\frac{2 \cdot C_{O_2} \cdot D_{O_2}}{q_{O_2} \cdot \rho^{exp}_{granule}}} \tag{8.13}$$

where D_{O2} is the oxygen diffusivity ($dm^2\ d^{-1}$), S_{O2} is the oxygen concentration in the liquid phase (g $O_2\ L^{-1}$), q_{O2} is the specific oxygen uptake rate (g $O_2\ g^{-1}$ VSS d^{-1}), $\rho^{exp}_{granule}$ is the granule density (g VSS $(L_{granule})^{-1}$) and δ is the oxygen penetration depth (dm).

To estimate the value of the oxygen uptake rate (q_{O2}), an iterative method was used. Initially, the q_{O2} was calculated as the oxygen required to fully oxidize the acetate fed and to convert ammonium to nitrate in each case. According to Eq. 8.13, an oxygen penetration depth was estimated. With this value, a fraction of active biomass was calculated according to Eq. 8.14.

$$\text{fraction of active biomass} = \frac{\frac{4}{3} \cdot \pi \cdot R_m^3 - \frac{4}{3} \cdot \pi \cdot (R_m - \delta)^3}{\frac{4}{3} \cdot \pi \cdot R_m^3} \tag{8.14}$$

Since the oxygen consumption rate can only be attributed to active biomass, a new q_{O2} value was calculated dividing the initial value by the percentage of active biomass calculated from Eq. 8.14. With this new q_{O2} value, the oxygen penetration depth and the fraction of active biomass were recalculated and this iterative process was repeated until convergence. The obtained values of q_{O2} were: 5.6; 1.0 and 0.7 g O_2 (g VSS)$^{-1}$ d^{-1} and oxygen penetration depth of: 0.15; 0.35 and 0.42 mm for COD/N ratios of 0; 1.25 and 5.5 g g^{-1}, respectively.

Biofilm porosity is implicitly fixed in AQUASIM software when the modeller inputs the initial composition of the biofilm. This initial value of porosity remains constant during the simulation time if the rate (of change) of porosity is set to zero. Further, the porosity value is the same along the granule radius in previous biofilm models developed for aerobic granules in previous contributions (Beun et al., 2001; de Kreuk et al., 2007), and

this of course has an impact on the estimation of both total biomass concentration and the density of the granules through the mathematical model.

The porosity for each discretization point (ε_i) along the granule radius was calculated according to Eq. 8.15.

$$\varepsilon_i = 1 - \left(\frac{X_H^i}{\rho_H} + \frac{X_{STO}^i}{\rho_{STO}} + \frac{X_A^i}{\rho_A} + \frac{X_N^i}{\rho_N} + \frac{X_I^i}{\rho_I} \right) \quad (8.15)$$

being ρ_H, ρ_{STO}, ρ_A, ρ_N and ρ_I the maximal densities of each type of solid fraction of the biomass in g COD $(L_{granule})^{-1}$.

As represented in Eq. 8.15, the porosity is dependent on the density of each solid fraction considered. A wide range of values for the density of different biomass types has been reported as presented in Table 8.4, de Kreuk et al. (2007) and Xavier et al., (2007) used a large value for the density of storage polymers in order to make their volume negligible compared to the active biomass. Lee and Park, (2007) used different densities for autotrophs and heterotrophs and varied the space occupancy of each biomass type in order to do not initially assume a value of porosity and therefore obtaining a prediction of porosity as an output of the model. In the present study, the values for the maximal density of each solids fraction were chosen as presented in Table 8.4.

Table 8.4. Maximum density of the different solids fractions (all values in g COD $(L_{biomass})^{-1}$) from literature reports. (ρ_A: maximum density of AOB; ρ_N: maximum density of NOB; ρ_H: maximum density of heterotrophs; ρ_{STO}: maximum density of storage products; ρ_I: maximum density of inert biomass)

ρ_A, ρ_N	ρ_H	ρ_{STO}	ρ_I	Reference
10	-	-	-	Downing and Nerenberg, (2008)
160	160	-	160	Montras et al., (2008)
100	100	-	100	Elenter et al., (2007)
350	350	$1 \cdot 10^5$	350	de Kreuk et al., (2007)
150	150	$1 \cdot 10^5$	150	Xavier et al., (2007)
450	200	-	450	Lee and Park, (2007)
350	150	350	150	This study

The initial concentrations of the biomass in the granule were fixed as follows (expressed as g COD L^{-1}): X_H = 1.0, X_A = 0.1, X_N = 0.1, X_{STO} = 0. The remaining value, i.e., the concentration of X_I in each discretization point was obtained with Eq. 8.15 once the porosity profile was fixed.

8.4.6.1 Detachment rate

The feast and famine periods occurring in each one of the cycles of the SBR (when organic matter is available in the influent, i.e., this is not applicable when COD/N=0) will result in small cyclic variations of the granule size. During the feast period, the granule radius will increase due to the accumulation of storage compounds inside the bacteria and then it will decrease during the famine period due to the consumption of those compounds (see Fig. 8.2).

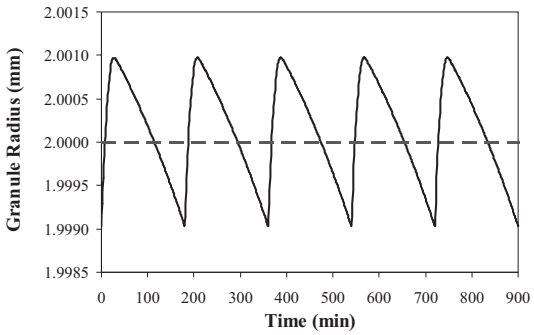

Figure 8.2. Comparison of the time course variations of the granule radius at COD/N =5.5 kg kg^{-1} obtained with the model developed in this study (—) and when assuming a ρ_{STO} = 1·10^5 g COD ($L_{granule}$)$^{-1}$ as in de Kreuk et al., (2007) (— — —).

Literature reports show how for modelling purposes, in order to make these cyclic variations in granule size negligible along the cycle it is recommended to use a very large value of ρ_{STO} as it has been done by de Kreuk et al., (2007) (see table 8.3) using a value of 1·10^5 g COD ($L_{granule}$)$^{-1}$ (Fig. 8.2). This is a powerful approach to easy the steady state of the biofilm thickness. Nevertheless, in the present study, ρ_{STO} was fixed at 350 g COD ($L_{granule}$)$^{-1}$, which probably describes more in detail what is happening in real systems. Due to this, a specific expression for the detachment rate in order to allow the achievement of steady state granule size was developed. A detachment rate (u_{Det}) was introduced when the growth velocity of granules was positive (Eq. 8.16, see Fig. 8.2).

$$u_{Det} = \left(\frac{(r - R_m)}{R_m} \cdot a + b_{Det} \right) \cdot u_F, \quad if\ u_F > 0,\ otherwise \quad u_{Det} = 0 \tag{8.16}$$

Being u_F the growth velocity of the granule (dm d^{-1}), r the simulated granules radius (dm), R_m the experimental mean radius (dm), a corresponds to a scaling factor fixed as 100. The parameter b_{Det} is a detachment coefficient that was tuned for each case to match the experimental R_m in steady state and its values were: 0.85, 0.9 and 0.95 for COD/N ratios of 5.5, 1.25 and 0 g g^{-1}, respectively. The first term in the sum in Eq. 8.16 is required since a variable detachment rate is needed to compensate the variations in the radius of the granule as presented in Fig. 8.2. When the radius of the granule was close to the maximum value of the radius ($r \sim R_m$) the first term of the sum in Eq. 8.16 was approximately zero and then the u_{Det} was equal to the product: $b_{Det} \cdot u_F$. Eq. 8.16 is an interesting way of describing detachment in this type of systems since it reproduces the cyclic variations in granule size allowing at the same time steady state conditions.

8.5. Results and discussion

8.5.1. Model validation at different COD/N ratios

In order to validate the proposed model, three different operational conditions characterized by different COD/N ratios were simulated to reach steady state conditions. Experimental data of ammonium, nitrite, nitrate, alkalinity, COD, total solids concentration and density of the granules were taken into account for the model validation. Results obtained from the model at steady state conditions were compared to the experimental data during an operational cycle for each COD/N ratio tested (Fig. 8.3, a-f).

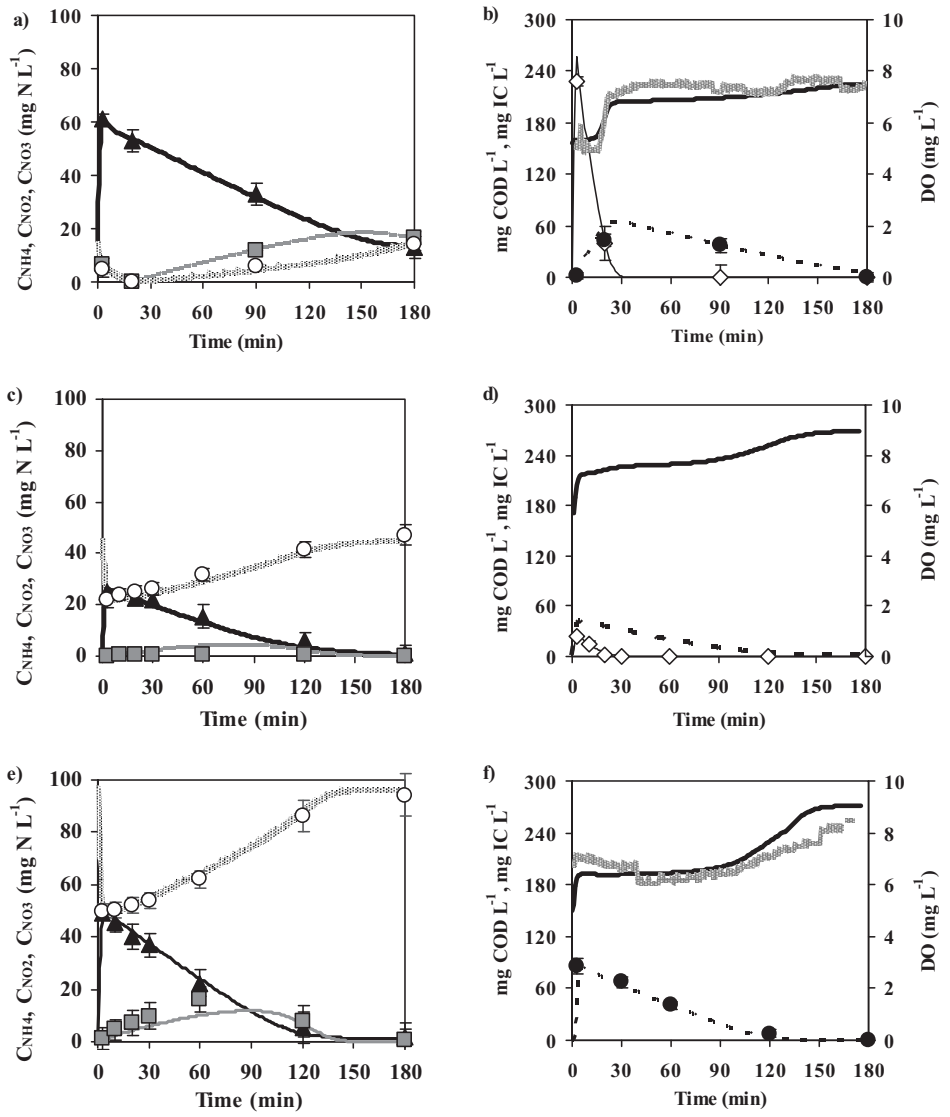

Figure 8.3. Time course variations of the experimental and simulated concentrations of NH_4^+-N (▲, ▬), NO_2^--N (■, ▬), NO_3^--N (○, ▭), DO (▭, ▬), IC (●, - - -) and COD (◇, ▬) in the bulk liquid of the granular SBR for the tested COD/N ratios: 5 g g^{-1} (a, b); 1.25 g g^{-1} (c, d) and 0 g g^{-1} (e, f) during a SBR cycle.

At a COD/N ratio of 5.5 g g^{-1}, the COD removal from the liquid phase during the feast phase, occurring in the first 20 minutes of each cycle, was accurately described by the model and fitted to the time course variation of the DO concentration and to the length of the denitrification. During the famine phase, the profiles of amxmonium, nitrite, nitrate and DO concentrations were in agreement with modelling predictions. Nitrite accumulation at the end of the cycle was also well predicted by the model. At a COD/N ratio of 1.25 g g^{-1}, the

experimental COD removal was well described by the model. However, a slight nitrite accumulation reaching 5 mg N L^{-1} was predicted by the model but it was not observed experimentally. Finally, at a COD/N ration of 0 g g^{-1}, all the nitrogenous compounds concentrations were accurately predicted as well as the time course variation of DO concentration. The accurate description of the soluble compounds by the validated model had an additional worth due to the fact that the kinetic and stoichiometric parameters of the model were obtained from the literature (see Appendix B for more details). Comparatively, a significant number of kinetic and stoichiometric parameters were estimated or calibrated in other previous GSBR published models (Su and Yu, 2006a; de Kreuk et al., 2007; Ni et al., 2008). Moreover, this is the first model for granular biomass validated with several experiments with different COD/N ratio in the influent.

On the other hand, Table 8.5 shows that the total solids concentration in the SBR predicted by the model for each COD/N agreed with the experimental values. Regarding the density of the granules similar values were obtained with the model compared to those obtained experimentally (Table 8.5). The accurate description of the experimental values of the total solids concentration and the density of the granules by the developed model was the most significant result of this work because little attention has been paid to this aspect in most of the previous published 1-D biofilm models for granular biomass (e.g., Su and Yu, 2006a; de Kreuk et al., 2007; Ni et al., 2008).

Table 8.5. Experimental and predicted total solids concentration (g VSS L^{-1}) and density of the granules (g VSS $(L_{granule})^{-1}$) in the reactor for each tested COD/N ratio.

COD/N g g^{-1}	X_{total}^{model}	X_{total}^{exp}	$\rho_{granule}^{model}$	$\rho_{granule}^{exp}$
5.50	5.2	5.0 ± 1.5	24	23 ± 10
1.25	1.9	2.0 ± 0.5	22	23 ± 10
0.00	1.0	1.0 ± 0.4	24	23 ± 10

8.5.1.1. The role of alkalinity

As it is shown in the Fig 8.3a and 8.3b ammonium was not limiting at any point along the operational cycle, however, the ammonium oxidizing rate decreased at a certain moment due to the low alkalinity concentration. Data from Table 8.1 show that no alkalinity was added in the influent for a COD/N ratio of 5.5 g g^{-1}. Alkalinity was accumulated in the media due to the COD consumption in the bulk liquid during the first 30 minutes of the cycle (feast period). Alkalinity production was mainly due to: 1) storage of acetate which released an equivalent molar amount of alkalinity to meet the electrochemical balance 2) occurrence of denitrification (Appendixes C and D). During the rest of the cycle, alkalinity was mainly consumed by AOB for the oxidation of ammonium to nitrite. When the alkalinity dropped below 12 mg C L^{-1}, which corresponded to the double of the half saturation constant for alkalinity, the ammonium oxidation rate decreased.

In the case of COD/N of 1.25 g g^{-1}, external addition of alkalinity as sodium bicarbonate (Table 8.1) was mandatory to achieve full ammonium oxidation. In order to corroborate the necessity of alkalinity supply, a cycle was performed without addition of alkalinity in the influent (Fig. 8.4). It was observed that the alkalinity concentration reached only a maximal value of 3 mg IC L^{-1} at the end of the feast period. The amount of ammonium oxidized was only 4 mg N L^{-1} whereas, when adding alkalinity in the influent, full ammonium oxidation was reached (Fig. 8.3c and 8.3d).

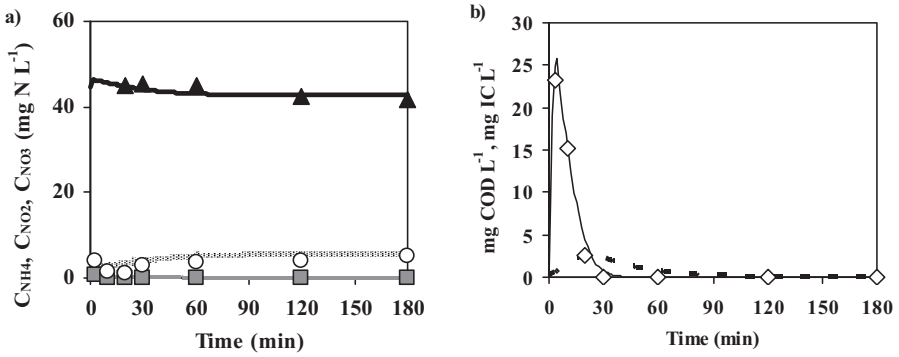

Figure 8.4. Time course variations of the experimental (symbol) and simulated (line) concentrations of NH_4^+-N (▲, ▬), NO_2^--N (■, ▬), NO_3^--N (○, ▬), IC (– – –) and COD (◇, ▬) in the bulk liquid of the granular SBR for 1.25 g g^{-1} (a, b) without external addition of alkalinity.

In the case of COD/N of 0 g g^{-1}, again, external addition of alkalinity is mandatory in order to not limit ammonium oxidation.

8.5.2. Distribution of the particulate compounds at different COD/N ratios

The concentrations of each particulate compound fraction in the SBR were obtained by simulation according to Eq. 8.10 (Table 8.6). According to the simulations, the percentage of autotrophs (X_A + X_N) was only 5% of the total active biomass (X_A + X_N + X_H) when a COD/N ratio of 5.5 g g^{-1} was applied while it increased up to 30% when the COD/N ratio was 1.25 g g^{-1} and finally up to 100% when the COD/N ratio was 0 kg kg^{-1}. The X_A/X_N ratio predicted by the model was close to 2 g COD-X_A g^{-1} COD-X_N for the low COD/N ratios (0 and 1.25 g g^{-1}). However, for the COD/N ratio of 5.5 g g^{-1}, the predicted ratio reached a value of 5.3 g COD-X_A g^{-1} COD-X_N which resulted in the prediction of nitrite accumulation in the reactor due to the lower oxidation rate of NOB (three times lower) in comparison to AOB.

Table 8.6. Predicted concentrations of the different particulate compounds (kg VSS m^{-3}) in the SBR for each simulated COD/N ratio.

COD/N	X_A	X_N	X_H	X_{STO}	X_I	X_{total}^{model}
g g^{-1}	g VSS L^{-1}					
5.50	0.07	0.01	1.47	0.21	3.47	5.23
1.25	0.07	0.03	0.20	0.02	1.59	1.91
0.00	0.12	0.06	0.00	0.00	0.83	1.01

The percentages of inert particulate organic material (X_I) were 60%, 80% and 80% of the total solids concentration corresponding to the COD/N ratios of 5.5, 1.25 and 0 g g^{-1}, respectively.

The modelling results indicated that the fraction X_{STO} was larger when the feeding media contained the highest COD/N ratio and this fact is in agreement with the expectations. The value of the X_{STO}/X_H ratio obtained at the end of the cycle for COD/N ratios of 5.5 and 1.25 was 0.14 g g^{-1} in both cases.

8.5.3. Predicted profiles of particulate compounds and DO concentrations inside the granules.

The particulate compounds distribution and DO concentration depth profiles inside the granules were obtained by simulation (Fig. 8.5, a-f). These profiles show that the autotrophic biomass (X_A and X_N) as well as the PHB content (X_{STO}) were present only in the external layers of the granules in the three simulated COD/N ratios. The reason of this distribution is that some substrates were available only in the most external part of the granule.

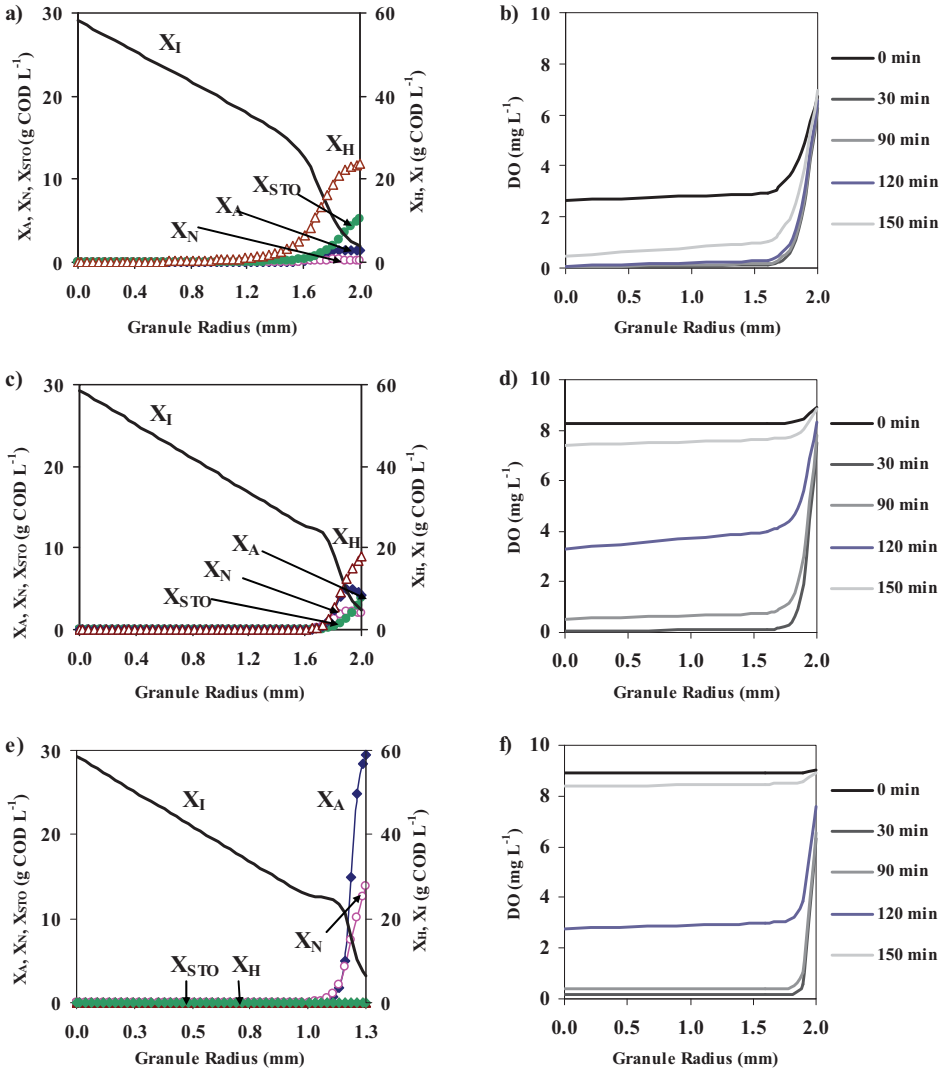

Figure 8.5. a), c), e) Depth profiles of particulate compounds and b), d), f) DO concentration depth profiles in the granules as simulated with the model at different cycle times: a), b) COD/N = 5.5 g g^{-1} c), d) COD/N = 1.25 g g^{-1} e), f) COD/N = 0 g g^{-1}. The radius is equal to zero at the centre of the granule.

Two different periods could be defined during a cycle of the GSBR operated with a COD/N ratio of 5.5 g g^{-1}. The first 20 minutes corresponded to the feast phase and the main processes of this period were the oxidation of acetate and the accumulation of storage products by heterotrophs. The rest of the cycle corresponded to the famine phase and the main process of this period was the oxidation of ammonium and nitrite by autotrophs. The DO concentration depth profile in the granule obtained by simulation (Fig. 8.5b), demonstrates that the oxygen penetration increased as heterotrophs became limited by acetate concentration. The model predicts that the oxygen only penetrated 0.35 mm during the feast phase due to the high oxygen uptake rate of the heterotrophic biomass. The maximal DO concentration reached in the centre of the granule was slightly higher than 2 mg L^{-1} but only for few minutes remaining 80% of the granule in anoxic conditions during a large part of the cycle. The denitrification of the nitrate left at the end of the previous cycle was performed during the first minutes of the cycle in this anoxic zone. The denitrification process occurred significantly only in the experiment corresponding to the COD/N = 5.5 g g^{-1} and the amount of denitrifying biomass can not be estimated independently from the heterotrophic biomass performing the organic matter oxidation.

Heterotrophic biomass (X_H) was present in the external layers when the COD/N ratio was low (1.25 g g^{-1}) but they were present in a wider zone of the granule depth (i.e., more spread) when the COD/N ratio increased up to 5 g g^{-1} since they could grow in the anoxic zone carrying out the denitrification process.

On the other hand, the concentration of autotrophic biomasses (X_A + X_N) decreased along the granule depth when the COD/N ratio was 0 g g^{-1} due to the decrease in the DO concentration caused by the nitrifying activity (Fig. 8.5 e). However, according to the model predictions, when the COD/N ratio increased, the concentrations of autotrophs registered a maximal peak deeper in the granule due to the competition with the heterotrophs for the DO in the external layers of the granule (Fig. 8.5 b). From the results it is also observed that the AOB are always present in higher concentrations than the NOB due to the higher growth rate, yield and affinity for oxygen of the former compared to the latter (Appendix B).

With regard to the oxygen penetration depth, its values were 0.30 and 0.12 mm when the COD/N ratio were 1.25 and 0 kg kg^{-1} respectively, and they are in agreement with the values initially estimated with Eq. 8.14. In the three simulated COD/N ratios, the concentration of inert particulate organic material (X_I) increased progressively in direction to the centre of the granules indicating the presence of an important fraction of the granule which is not active.

8.5.3.1 Evolution of the storage compounds along the cycle for COD/N = 5.5

The cyclic variation of the X_{STO} concentration in the granules represented in Fig. 8.6. The concentration of X_{sto} increases during the feast period (form minute 0 to minute 30) and decreases progressively during the famine period (from minute 30 to the end of the cycle). Results showed that storage products were present during the whole cycle until a depth around 0.5 mm inside the granule corresponding to the aerobic and anoxic accumulation during the feast period. Therefore, denitrification was still possible during the famine phase using the storage compounds (simultaneous nitrification/denitrification) as already demonstrated by Beun et al., (2001).

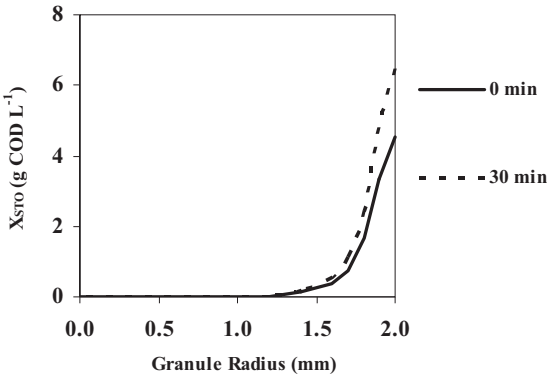

Figure 8.6. X_{STO} concentration profile along the granule at time 0 (—) and 30 min (- - -) for a COD/N ratio equal to 5.5 g g^{-1}. The radius is equal to zero at the centre of the granule.

8.6. Conclusions

● A one dimensional model was developed and evaluated to describe a granular SBR at variable COD/N ratios. The consideration of the nitrification as a two-step process and also the simultaneous growth and storage of organic substrates by heterotrophic bacteria were the main two differences of the developed model with regards to the conventional biological processes description in the ASM-type models.

● The simulation of steady state conditions of the GSBR operating at three different influent COD/N ratios was successfully carried out. An accurate description of the experimental results of the GSBR operation with regards to soluble compounds concentrations, but also biomass density and total solids concentration, were obtained using stoichiometric and kinetic parameters from the literature.

● The key factors which allowed the successful description of the biomass characteristics were: (i) the definition of different densities for heterotrophs and autotrophs and (ii) the introduction of a porosity profile along the granule depth, in which the porosity of the inner (anoxic) zone of the granule was lower than the one in the outer (aerobic) shell.

● Nitrite build up and alkalinity profile along the cycle were also accurately represented by the model. DO profiles inside the granules revealed that nitrite build up was possible even by working at high DO in the bulk liquid. External additions of alkalinity were mandatory to achieve full ammonium oxidation for COD/N ratios of 1.25 and 0 g g^{-1}.

● The model predicted that the largest percentage of particulate compounds in the granules corresponded to the inert particulate organic material (60-80%). On the other hand, the model predicted that the presence of active biomass (heterotrophs or nitrifiers) in the granules obtained with low COD/N ratios (1.25 and 0 g g^{-1}) was restricted to the most external layers of the granules. However, the presence of active biomass in the granules obtained with a high COD/N ratio (5.5 g g^{-1}) achieved more internal layers (more than twice comparing with the low COD/N ratios).

● This model will allow developing the knowledge on aerobic granules grown in SBR in order to analyse and design pilot and full scale plants.

8.7. References

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APPENDIX A: Kinetic equations

Process	Equation
1. Aeration	$k_L a \left(C^*_{O2} - C_{O2}\right)$
Heterotrophic bacteria	
2. Aerobic growth on C_S	$\mu_{H,max} \frac{C_{O2}}{K^H_{O2} + C_{O2}} \frac{C_S}{K_{Ss} + C_S} X_H$
3. Aerobic growth on X_{STO}	$\mu_{H,max} \frac{C_{O2}}{K^H_{O2} + C_{O2}} \frac{C_S}{K_{Ss} + C_S} \frac{X_{STO}/X_H}{K_{STO} + X_{STO}/X_H} X_H$
4. Anoxic growth on S_S with C_{NO2}	$\mu_{H,max}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO2}}{K^H_{NO2} + C_{NO2}} \frac{C_S}{K_{Ss} + C_S} X_H$
5. Anoxic growth on S_S with C_{NO3}	$\mu_{H,max}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO3}}{K^H_{NO3} + C_{NO3}} \frac{C_S}{K_{Ss} + C_S} X_H$
6. Anoxic growth on X_{STO} with C_{NO2}	$\mu_{H,max}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO2}}{K^H_{NO2} + C_{NO2}} \frac{X_{STO}/X_H}{K_{STO} + X_{STO}/X_H} X_H$
7. Anoxic growth on X_{STO} with C_{NO3}	$\mu_{H,max}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO3}}{K^H_{NO3} + C_{NO3}} \frac{X_{STO}/X_H}{K_{STO} + X_{STO}/X_H} X_H$
8. Aerobic endogenous resp.	$b_H \frac{C_{O2}}{K^H_{O2} + C_{O2}} X_H$
9. Anoxic endogenous resp. on C_{NO2}	$b_H\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO2}}{K^H_{NO2} + C_{NO2}} X_H$
10. Anoxic endogenous resp. on C_{NO3}	$b_H\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO3}}{K^H_{NO3} + C_{NO3}} X_H$
11. Aerobic formation of X_{STO}	$k_{STO} \frac{C_{O2}}{K^H_{O2} + C_{O2}} \frac{C_S}{K_{Ss} + C_S} X_H$
12. Anoxic formation of X_{STO} on C_{NO2}	$k_{STO} \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO2}}{K^H_{NO2} + C_{NO2}} \frac{C_S}{K_{Ss} + C_S} X_H$
13. Anoxic formation of X_{STO} on C_{NO3}	$k_{STO}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO3}}{K^H_{NO3} + C_{NO3}} \frac{C_S}{K_{Ss} + C_S} X_H$
14. Aerobic resp. of X_{STO}	$b_{STO} \frac{C_{O2}}{K^H_{O2} + C_{O2}} X_{STO}$
15. Anoxic resp. of X_{STO} on C_{NO2}	$b_{STO}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO2}}{K^H_{NO2} + C_{NO2}} X_{STO}$
16. Anoxic resp. of X_{STO} on C_{NO3}	$b_{STO}\, \eta_g \frac{K^H_{O2}}{K^H_{O2} + C_{O2}} \frac{C_{NO3}}{K^H_{NO3} + C_{NO3}} X_{STO}$
Ammonia-oxidizing bacteria (AOB)	
17. Aerobic growth	$\mu_{A,max} \frac{C_{O2}}{K^A_{O2} + C_{O2}} \frac{C_{NH4}}{K_{NH4} + C_{NH4}} \frac{C_{ALK}}{K_{ALK} + C_{ALK}} X_A$
18. Aerobic endogenous resp.	$b_A \frac{C_{O2}}{K^A_{O2} + C_{O2}} X_A$
19. Anoxic endogenous resp. on C_{NO2}	$b_A\, \eta_g \frac{K^A_{O2}}{K^A_{O2} + C_{O2}} \frac{C_{NO2}}{K^A_{NO2} + C_{NO2}} X_A$
20. Anoxic endogenous resp. on C_{NO3}	$b_A\, \eta_g \frac{K^A_{O2}}{K^A_{O2} + C_{O2}} \frac{C_{NO3}}{K^A_{NO3} + C_{NO3}} X_A$
Nitrite-oxidizing bacteria (NOB)	
21. Aerobic growth	$\mu_{N,max} \frac{C_{O2}}{K^N_{O2} + C_{O2}} \frac{C_{NO2}}{K^N_{NO2} + C_{NO2}} X_N$
22. Aerobic endogenous resp.	$b_N \frac{C_{O2}}{K^N_{O2} + C_{O2}} X_N$
23. Anoxic endogenous resp. on C_{NO2}	$b_N\, \eta_g \frac{K^N_{O2}}{K^N_{O2} + C_{O2}} \frac{C_{NO2}}{K^N_{NO2} + C_{NO2}} X_N$
24. Anoxic endogenous resp. on C_{NO3}	$b_N\, \eta_g \frac{K^N_{O2}}{K^N_{O2} + C_{O2}} \frac{C_{NO3}}{K^N_{NO3} + C_{NO3}} X_N$

APPENDIX B: Kinetic parameters

Symbol	Definition	Value	Units	Ref.
b_A	Aerobic endogenous resp. rate of X_A	0.18	d^{-1}	[1]
b_N	Aerobic endogenous resp. rate of X_N	0.16	d^{-1}	[1]
b_H	Aerobic endogenous resp. rate ofX_H	0.26	d^{-1}	[2]
b_{STO}	Aerobic resp. rate for X_{STO}	0.26	d^{-1}	[2]
C^*_{O2}	Saturation oxygen concentration	9	mg O_2 L^{-1}	Equilibrium data
D_{NH4}	Diffusion coefficient of C_{NH4} in water	$1.61 \cdot 10^{-4}$	m^2 d^{-1}	[3]
D_{NO2}	Diffusion coefficient of C_{NO2} in water	$1.47 \cdot 10^{-4}$	m^2 d^{-1}	[3]
D_{NO3}	Diffusion coefficient of C_{NO3} in water	$1.47 \cdot 10^{-4}$	m^2 d^{-1}	[3]
D_{O2}	Diffusion coefficient of C_{O2} in water	$1.72 \cdot 10^{-4}$	m^2 d^{-1}	[3]
D_{Ss}	Diffusion coefficient for C_S	$1.00 \cdot 10^{-4}$	m^2 d^{-1}	[4]
f_X	Production of X_I in endogenous resp.	0.2	-	[2]
$i_{N,Xi}$	N content of X_I	0.04	g N (g COD-X_i)$^{-1}$	[2]
$i_{N,BM}$	N content of biomass, X_H, X_A, X_N	0.08	g N (g COD-X)$^{-1}$	[2]
η_g	Reduction factor for anoxic activity	0.6	-	[2]
K_{Alk}	Saturation constant for C_{Alk}	6	mg IC L^{-1}	[2]
k_La	Oxygen mass transfer coefficient	1008	d^{-1}	Measured
K_{STO}	Saturation constant for X_{STO}	1.0	g COD-X_{STO} (g COD-X_H)$^{-1}$	[2]
K_{Ss}	Saturation constant for C_S	0.004	g COD L^{-1}	[2]
K^A_{NH4}	C_{NH4} saturation constant for X_A	1.0	mg NH_4^+-N L^{-1}	[2]
K^A_{NO2}	C_{NO2} saturation constant for X_A	1.0	mg NO_2^--N L^{-1}	[2]
K^A_{NO3}	C_{NO3} saturation constant for X_A	1.0	mg NO_3^--N L^{-1}	[2]
K^N_{NO2}	C_{NO2} saturation constant for X_N	1.0	mg NO_2^--N L^{-1}	[2]
K^N_{NO3}	C_{NO3} saturation constant for X_N	1.0	mg NO_3^--N L^{-1}	[2]
K^H_{NO2}	C_{NO2} saturation constant for X_H	0.5	mg NO_2^--N L^{-1}	[2]
K^H_{NO3}	C_{NO3} saturation constant for X_H	0.5	mg NO_3^--N L^{-1}	[2]
K^A_{O2}	C_{O2} saturation constant for X_A	0.74	mg O_2 L^{-1}	[5]
K^N_{O2}	C_{O2} saturation constant for X_N	1.75	mg O_2 L^{-1}	[5]
K^H_{O2}	C_{O2} saturation constant for X_H	0.2	mg O_2 L^{-1}	[2]
$\mu_{A,max}$	Maximum growth rate of X_A	1.10	d^{-1}	[1]
$\mu_{N,max}$	Maximum growth rate of X_N	0.96	d^{-1}	[1]
$\mu_{H,max}$	Maximum growth rate of X_H	2.6	d^{-1}	[2]
k_{STO}	Storage rate constant	6.6	d^{-1}	[2]
ρ_A	Maximum density for X_A	350	g COD L^{-1}	[6]
ρ_N	Maximum density of X_N	350	g COD L^{-1}	[6]
ρ_H	Maximum density for X_H	150	g COD L^{-1}	[7]
ρ_{STO}	Maximum density of X_{STO}	350	g COD L^{-1}	Assumed
ρ_I	Maximum density of X_I	150	g COD L^{-1}	[7]
Y_A	Yield of X_A per C_{NH4}	0.18	g COD-X_A (g N)$^{-1}$	[1]
Y_N	Yield of X_N per C_{NO2}	0.08	g COD-X_N (g N)$^{-1}$	[1]
Y_H	Yield of X_H per C_S	0.57	g COD-X_H (g COD-S_S)$^{-1}$	[8]
Y_{HSTO}	Yield of X_H per X_{STO}	0.68	g COD-X_H (g COD-X_{STO})$^{-1}$	[8]
Y_{STO}	Yield coefficient for storage on substrate	0.80	g COD-X_{STO} (g COD-S_S)$^{-1}$	[8]

[1] Jubany et al., 2008 [2] Henze et al., 2000 [3] Picioreanu et al., 1997 [4] Perez et al., 2005 [5] Guisasola et al., 2005 [6] de Kreuk et al., 2007 [7] Xavier et al., 2007 [8] Sin et al., 2005.

APPENDIX C: Stoichiometric matrix of soluble and particulate components

	C_S COD g L^{-1}	C_{Alk} Alk mol L^{-1}	C_{O2} O_2 g L^{-1}	C_{NH4} N g L^{-1}	C_{NO2} N g L^{-1}	C_{NO3} N g L^{-1}	C_{N2} N g L^{-1}	X_H COD g L^{-1}	X_{STO} COD g L^{-1}	X_A COD g L^{-1}	X_N COD g L^{-1}	X_I COD g L^{-1}
Charge	-1	-1	0	+1	-1	-1	-1	0	0	0	0	0
1. Aeration	0	0	1	0	0	0	0	0	0	0	0	0
Heterotrophic bacteria												
2. Aerobic growth on C_S	$-1/Y_H$	$1/(64\cdot Y_H)-i_B/14$	$-(1/Y_H-1)$	$-i_B$	0	0	0	+1	0	0	0	0
3. Aerobic growth on X_{STO}	0	$-i_B/14$	$-(1/Y_{HSTO}-1)$	$-i_B$	0	0	0	+1	$-1/Y_{HSTO}$	0	0	0
4. Anoxic growth on S_S with C_{NO2}	$-1/Y_H$	$1/(64\cdot Y_H)+(1/Y_H-1)/24-i_B/14$	0	$-i_B$	$-(1/Y_H-1)/1.71$	0	$(1/Y_H-1)/1.71$	+1	0	0	0	0
5. Anoxic growth on S_S with C_{NO3}	$-1/Y_H$	$1/(64\cdot Y_H)+(1/Y_H-1)/40-i_B/14$	0	$-i_B$	0	$-(1/Y_H-1)/2.86$	$(1/Y_H-1)/2.86$	+1	0	0	0	0
6. Anoxic growth on X_{STO} with C_{NO2}	0	$(1/Y_{HSTO}-1)/24-i_B/14$	0	$-i_B$	$-(1/Y_{HSTO}-1)/1.71$	0	$(1/Y_{HSTO}-1)/1.71$	+1	$-1/Y_{HSTO}$	0	0	0
7. Anoxic growth on X_{STO} with C_{NO3}	0	$(1/Y_{HSTO}-1)/40-i_B/14$	0	$-i_B$	0	$-(1/Y_{HSTO}-1)/2.86$	$(1/Y_{HSTO}-1)/2.86$	+1	$-1/Y_{HSTO}$	0	0	0
8. Aerobic endogenous resp.	0	$(i_B-i_{XI}\cdot f_X)/14$	$-(1-f_X)$	$i_B-i_{XI}\cdot f_X$	0	0	0	-1	0	0	0	f_X
9. Anoxic endogenous resp. on C_{NO2}	0	$(i_B-i_{XI}\cdot f_X)/14+(1-f_X)/24$	0	$i_B-i_{XI}\cdot f_X$	$-(1-f_X)/1.71$	0	$(1-f_X)/1.71$	-1	0	0	0	f_X
10. Anoxic endogenous resp. on C_{NO3}	0	$(i_B-i_{XI}\cdot f_X)/14+(1-f_X)/40$	0	$i_B-i_{XI}\cdot f_X$	0	$-(1-f_X)/2.86$	$(1-f_X)/2.86$	-1	0	0	0	f_X
11. Aerobic formation of X_{STO}	$-1/Y_{STO}$	$1/(64\cdot Y_{STO})$	$-(1/Y_{STO}-1)$	0	0	0	0	0	+1	0	0	0
12. Anoxic formation of X_{STO} on C_{NO2}	$-1/Y_{STO}$	$1/(64\cdot Y_{STO})+(1/Y_{STO}-1)/24$	0	0	$-(1/Y_{STO}-1)/1.71$	0	$(1/Y_{STO}-1)/1.71$	0	+1	0	0	0
13. Anoxic formation of X_{STO} on C_{NO3}	$-1/Y_{STO}$	$1/(64\cdot Y_{STO})+(1/Y_{STO}-1)/40$	0	0	0	$-(1/Y_{STO}-1)/2.86$	$(1/Y_{STO}-1)/2.86$	0	+1	0	0	0
14. Aerobic resp. of X_{STO}	0	0	-1	0	0	0	0	0	-1	0	0	0
15. Anoxic resp. of X_{STO} on C_{NO2}	0	1/24	0	0	-1/1.71	0	1/1.71	0	-1	0	0	0
16. Anoxic resp. of X_{STO} on C_{NO3}	0	1/40	0	0	0	-1/2.86	1/2.86	0	-1	0	0	0
Ammonia-oxidizing bacteria												
17. Aerobic growth	0	$-(i_B+2/Y_A)/14$	$-(3.43/Y_A-1)$	$-(i_B+1/Y_A)$	$1/Y_A$	0	0	0	0	1	0	0
18. Aerobic endogenous resp.	0	$(i_B-i_{XI}\cdot f_X)/14$	$-(1-f_X)$	$i_B-i_{XI}\cdot f_X$	0	0	0	0	0	-1	0	f_X
19. Anoxic endogenous resp. on C_{NO2}	0	$(i_B-i_{XI}\cdot f_X)/14+(1-f_X)/24$	0	$i_B-i_{XI}\cdot f_X$	$-(1-f_X)/1.71$	0	$(1-f_X)/1.71$	0	0	-1	0	f_X
20. Anoxic endogenous resp. on C_{NO3}	0	$(i_B-i_{XI}\cdot f_X)/14+(1-f_X)/40$	0	$i_B-i_{XI}\cdot f_X$	0	$-(1-f_X)/2.86$	$(1-f_X)/2.86$	0	0	-1	0	f_X
Nitrite-oxidizing bacteria												
21. Aerobic growth	0	$-i_B/14$	$-1.14/Y_N$	$-i_B$	$-1/Y_N$	$1/Y_N$	0	0	0	0	1	0
22. Aerobic endogenous resp.	0	$(i_B-i_{XI}\cdot f_X)/14$	$-(1-f_X)$	$i_B-i_{XI}\cdot f_X$	0	0	0	0	0	0	-1	f_X
23. Anoxic endogenous resp. on C_{NO2}	0	$(i_B-i_{XI}\cdot f_X)/14+(1-f_X)/24$	0	$i_B-i_{XI}\cdot f_X$	$-(1-f_X)/1.71$	0	$(1-f_X)/1.71$	0	0	0	-1	f_X
24. Anoxic endogenous resp. on C_{NO3}	0	$(i_B-i_{XI}\cdot f_X)/14+(1-f_X)/40$	0	$i_B-i_{XI}\cdot f_X$	0	$-(1-f_X)/2.86$	$(1-f_X)/2.86$	0	0	0	-1	f_X

APPENDIX D: Numeric stoichiometric matrix of soluble and particulate components

	C_S COD g L^{-1}	C_{Alk} Alk g IC L^{-1}	C_{O2} O_2 g L^{-1}	C_{NH4} N g L^{-1}	C_{NO2} N g L^{-1}	C_{NO3} N g L^{-1}	C_{N2} N g L^{-1}	X_H COD g L^{-1}	X_{STO} COD g L^{-1}	X_A COD g L^{-1}	X_N COD g L^{-1}	X_I COD g L^{-1}
1. Aeration	0	0	1	0	0	0		0	0	0	0	0
Heterotrophic bacteria												
2. Aerobic growth on C_S	-1.754	0.263	-0.754	-0.076	0	0	0	+1	0	0	0	0
3. Aerobic growth on X_{STO}	0	-0.065	-0.471	-0.076	0	0	0	+1	-1.471	0	0	0
4. Anoxic growth on S_S with C_{NO2}	-1.754	0.641	0	-0.076	-0.440	0	0.440	+1	0	0	0	0
5. Anoxic growth on S_S with C_{NO3}	-1.754	0.490	0	-0.076	0	-0.264	0.264	+1	0	0	0	0
6. Anoxic growth on X_{STO} with C_{NO2}	0	0.170	0	-0.076	-0.275	0	0.275	+1	-1.471	0	0	0
7. Anoxic growth on X_{STO} with C_{NO3}	0	0.076	0	-0.076	0	-0.165	0.165	+1	-1.471	0	0	0
8. Aerobic endogenous resp.	0	0.058	-0.800	0.068	0	0	0	-1	0	0	0	0.2
9. Anoxic endogenous resp. on C_{NO2}	0	0.458	0	0.068	-0.467	0	0.467	-1	0	0	0	0.2
10. Anoxic endogenous resp. on C_{NO3}	0	0.298	0	0.068	0	-0.280	0.280	-1	0	0	0	0.2
11. Aerobic formation of X_{STO}	-1.250	0.234	-0.250	0	0	0	0	0	+1	0	0	0
12. Anoxic formation of X_{STO} on C_{NO2}	-1.250	0.359	0	0	-0.146	0	0.146	0	+1	0	0	0
13. Anoxic formation of X_{STO} on C_{NO3}	-1.250	0.309	0	0	0	-0.088	0.088	0	+1	0	0	0
14. Aerobic resp. of X_{STO}	0	0	-1.000	0	0	0	0	0	-1	0	0	0
15. Anoxic resp. of X_{STO} on C_{NO2}	0	0.500	0	0	-0.583	0	0.583	0	-1	0	0	0
16. Anoxic resp. of X_{STO} on C_{NO3}	0	0.300	0	0	0	-0.350	0.350	0	-1	0	0	0
Ammonia-oxidizing bacteria												
17. Aerobic growth	0	-9.589	-18.056	-5.632	5.556	0	0	0	0	1	0	0
18. Aerobic endogenous resp.	0	0.058	-0.800	0.068	0	0	0	0	0	-1	0	0.2
19. Anoxic endogenous resp. on C_{NO2}	0	0.458	0	0.068	-0.467	0	0.467	0	0	-1	0	0.2
20. Anoxic endogenous resp. on C_{NO3}	0	0.298	0	0.068	0	-0.280	0.280	0	0	-1	0	0.2
Nitrite-oxidizing bacteria												
21. Aerobic growth	0	-0.065	-14.250	-0.076	-12.500	12.500	0	0	0	0	1	0
22. Aerobic endogenous resp.	0	0.058	-0.800	0.068	0	0	0	0	0	0	-1	0.2
23. Anoxic endogenous resp. on C_{NO2}	0	0.458	0	0.068	-0.467	0	0.467	0	0	0	-1	0.2
24. Anoxic endogenous resp. on C_{NO3}	0	0.298	0	0.068	0	-0.280	0.280	0	0	0	-1	0.2

APPENDIX E: Molar stoichiometrical equations of the different processes implemented in the model

Process	Equation
Heterotrophic bacteria	
2. Aerobic growth on C_S	$CH_3COO^- + 0.86\ O_2 + 0.20\ NH_4^+ \rightarrow 0.20\ C_{5.06}H_{9.33}O_{1.82}N + 0.57\ H_2O + 0.80\ HCO_3^- + 0.19\ CO_2$
3. Aerobic growth on X_{STO}	$C_2H_3O + 0.72\ O_2 + 0.27\ NH_4^+ + 0.27\ OH^- \rightarrow 0.27\ C_{5.06}H_{9.33}O_{1.82}N + 0.92\ H_2O + 0.65\ CO_2$
4. Anoxic growth on S_S with C_{NO2}	$CH_3COO^- + 1.15\ NO_2^- + 0.20\ NH_4^+ \rightarrow 0.20\ C_{5.06}H_{9.33}O_{1.82}N + 0.99\ HCO_3^- + 0.95\ OH^- + 0.57\ N_2$
5. Anoxic growth on S_S with C_{NO3}	$CH_3COO^- + 0.69\ NO_3^- + 0.20\ NH_4^+ \rightarrow 0.20\ C_{5.06}H_{9.33}O_{1.82}N + 0.99\ HCO_3^- + 0.50\ OH^- + 0.34\ N_2 + 0.22\ H_2O$
6. Anoxic growth on X_{STO} with C_{NO2}	$C_2H_3O + 0.96\ NO_2^- + 0.27\ NH_4^+ \rightarrow 0.27\ C_{5.06}H_{9.33}O_{1.82}N + 0.48\ N_2 + 0.44\ H_2O + 0.65\ HCO_3^- + 0.04\ OH^-$
7. Anoxic growth on X_{STO} with C_{NO3}	$C_2H_3O + 0.58\ NO_2^- + 0.27\ NH_4^+ \rightarrow 0.27\ C_{5.06}H_{9.33}O_{1.82}N + 0.29\ N_2 + 0.63\ H_2O + 0.31\ HCO_3^- + 0.34\ CO_2$
8. Aerobic endogenous resp.	$C_{5.06}H_{9.33}O_{1.82}N + 4.58\ O_2 \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 0.89\ NH_4^+ + 0.89\ HCO_3^- + 3.12\ CO_2 + 1.72\ H_2O$
9. Anoxic endogenous resp. on C_{NO2}	$C_{5.06}H_{9.33}O_{1.82}N + 6.11\ NO_2^- + 1.33\ H_2O \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 3.05\ N_2 + 0.89\ NH_4^+ + 4.01\ HCO_3^- + 2.99\ OH^-$
10. Anoxic endogenous resp. on C_{NO3}	$C_{5.06}H_{9.33}O_{1.82}N + 3.67\ NO_3^- + 0.11\ H_2O \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 1.83\ N_2 + 0.89\ NH_4^+ + 4.01\ HCO_3^- + 0.55\ OH^-$
11. Aerobic formation of X_{STO}	$CH_3COO^- + 0.40\ O_2 + 0.07\ H_2O \rightarrow 0.71\ C_2H_3O + 0.58\ HCO_3^- + 0.42\ OH^-$
12. Anoxic formation of X_{STO} on C_{NO2}	$CH_3COO^- + 0.53\ NO_2^- + 0.33\ H_2O \rightarrow 0.71\ C_2H_3O + 0.27\ N_2 + 0.58\ HCO_3^- + 0.96\ OH^-$
13. Anoxic formation of X_{STO} on C_{NO3}	$CH_3COO^- + 0.32\ NO_3^- + 0.23\ H_2O \rightarrow 0.71\ C_2H_3O + 0.16\ N_2 + 0.58\ HCO_3^- + 0.74\ OH^-$
14. Aerobic resp. of X_{STO}	$C_2H_3O + 2.25\ O_2 \rightarrow 2.00\ CO_2 + 1.50\ H_2O$
15. Anoxic resp. of X_{STO} on C_{NO2}	$C_2H_3O + 3.00\ NO_2^- \rightarrow 1.50\ N_2 + 2.00\ HCO_3^- + 1.00\ OH^-$
16. Anoxic resp. of X_{STO} on C_{NO3}	$C_2H_3O + 1.80\ NO_3^- \rightarrow 0.90\ N_2 + 1.80\ HCO_3^- + 0.60\ H_2O + 0.20\ CO_2$
Ammonia-oxidizing bacteria	
17. Aerobic growth	$NH_4^+ + 1.40\ O_2 + 1.99\ HCO_3^- \rightarrow 0.01\ C_{5.06}H_{9.33}O_{1.86}N + 0.99\ NO_2^- + 2.93\ H_2O + 1.92\ CO_2$
18. Aerobic endogenous resp.	$C_{5.06}H_{9.33}O_{1.82}N + 4.58\ O_2 \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 0.89\ NH_4^+ + 0.89\ HCO_3^- + 3.12\ CO_2 + 1.72\ H_2O$
19. Anoxic endogenous resp. on C_{NO2}	$C_{5.06}H_{9.33}O_{1.82}N + 6.11\ NO_2^- + 1.33\ H_2O \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 3.05\ N_2 + 0.89\ NH_4^+ + 4.01\ HCO_3^- + 2.99\ OH^-$
20. Anoxic endogenous resp. on C_{NO3}	$C_{5.06}H_{9.33}O_{1.82}N + 3.67\ NO_3^- + 0.11\ H_2O \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 1.83\ N_2 + 0.89\ NH_4^+ + 4.01\ HCO_3^- + 0.55\ OH^-$
Nitrite-oxidizing bacteria	
21. Aerobic growth	$NO_2^- + 0.47\ O_2 + 0.01\ NH_4^+ + 0.01\ HCO_3^- + 0.02\ CO_2 + 0.01\ H_2O \rightarrow 0.01\ C_{5.06}H_{9.33}O_{1.86}N + NO_3^-$
22. Aerobic endogenous resp.	$C_{5.06}H_{9.33}O_{1.82}N + 4.58\ O_2 \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 0.89\ NH_4^+ + 0.89\ HCO_3^- + 3.12\ CO_2 + 1.72\ H_2O$
23. Anoxic endogenous resp. on C_{NO2}	$C_{5.06}H_{9.33}O_{1.82}N + 6.11\ NO_2^- + 1.33\ H_2O \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 3.05\ N_2 + 0.89\ NH_4^+ + 4.01\ HCO_3^- + 2.99\ OH^-$
24. Anoxic endogenous resp. on C_{NO3}	$C_{5.06}H_{9.33}O_{1.82}N + 3.67\ NO_3^- + 0.11\ H_2O \rightarrow 0.22\ C_{4.74}H_{6.53}O_{1.63}N_{0.5} + 1.83\ N_2 + 0.89\ NH_4^+ + 4.01\ HCO_3^- + 0.55\ OH^-$

Conclusiones

Se presentan aquí las conclusiones generales de este trabajo de investigación, en el que se evaluaron y desarrollaron diferentes poblaciones bacterianas en agregados o gránulos para la eliminación autótrofa de nitrógeno.

1. Granulación de bacterias autótrofas

De los resultados obtenidos se concluye que es posible desarrollar las distintas poblaciones bacterianas involucradas en la eliminación autótrofa de nitrógeno (oxidantes de amonio, de nitrito y Anammox) en forma de gránulos utilizando reactores secuenciales (SBR: Sequencing batch reactor). Los gránulos oxidantes de amonio se obtuvieron a partir de gránulos originalmente heterótrofos, donde se eliminaba el nitrógeno mediante nitrificación-desnitrificación y en los que se ha ido retirando progresivamente la fuente de materia orgánica de la alimentación. Los gránulos oxidantes de nitrito fueron obtenidos a partir de gránulos nitrificantes, cambiando la fuente de nitrógeno en la alimentación de amonio a nitrito. En el caso de los gránulos Anammox, su desarrollo se llevó a cabo a través de la adición de cloruro sódico para fomentar la formación de un precipitado que favoreció la compactación de los gránulos formados. En todos los casos, las propiedades de sedimentación de los gránulos (índice volumétrico de lodos, densidad, tamaño de partícula, etc.) fueron suficientemente buenas para garantizar una óptima retención de la biomasa en el sistema.

2. Efectos de la concentración de oxígeno disuelto

El uso de biomasa granular implica la presencia de resistencias a la transferencia de materia, lo que provoca un gradiente de concentraciones en el interior del gránulo. En el caso de los sistemas aerobios, el transporte de oxígeno disuelto (OD) desde la fase líquida hacia el gránulo es generalmente la etapa limitante y, por lo tanto, la que marca la velocidad global del proceso. Esto permite operar el sistema en condiciones limitantes de oxígeno a pesar de tener concentraciones de OD altas en la fase líquida, lo que favorece el desarrollo de poblaciones bacterianas con una alta afinidad por ese sustrato.

Al alimentar el reactor con un medio autótrofo con amonio como fuente de nitrógeno, la limitación por OD causó que la población predominante en los gránulos fuese la oxidante de amonio dada su mayor afinidad por el oxígeno en comparación con la población oxidante de nitrito. Basándose en este principio ha sido posible la operación estable de un sistema granular nitrificante llevando a cabo la nitrificación parcial del 50% de amonio a nitrito a temperatura ambiente. Esto se consiguió en estas condiciones a pesar de que, a temperatura ambiente (alrededor de 20 ºC), las velocidades máximas de crecimiento de las bacterias oxidantes de nitrito (BON) son superiores a las de las bacterias oxidantes de amonio (BOA).

En el caso de poblaciones nitrito oxidantes, un aumento en el flujo de oxígeno hacia el gránulo causó la evolución de las poblaciones estrategas de la k (*Nitrospira*) hacia estrategas de la r (*Nitrobacter*).

3. Operación de sistemas CANON a temperatura ambiente

El hecho de tener una población de BOA en los gránulos aerobios que consume el oxígeno disuelto del medio para oxidar parcialmente el amonio a nitrito, permite la presencia de zonas anóxicas en el interior de los gránulos. En estas zonas anóxicas, las bacterias Anammox pueden crecer consumiendo el nitrito y el amonio sobrantes, llevando a cabo el proceso denominado CANON. En el caso del reactor SBR aireado en continuo en el que las concentraciones de OD variaban en el rango 2—4 mg O_2 L^{-1} se promovió la formación de

gránulos con las poblaciones estratificadas, de forma que las bacterias Anammox se colocaron en el interior mientras que las BOA se encontraban en las capas más externas. En cambio, en el SBR aireado en forma de pulsos, las concentraciones de OD en el medio fueron inferiores (entre 0,3 y 0,5 mg O_2 L^{-1}) y permitió que las bacterias Anammox crecieran fundamentalmente en forma de gránulos mientras que las AOB lo hicieron en forma de biomasa en suspensión.

La puesta en marcha del proceso CANON se realizó partiendo de un sistema de nitrificación parcial en el que se inoculó biomasa pobremente enriquecida en bacterias Anammox. Esto permitió el arranque del proceso CANON en un periodo de un mes disminuyendo significativamente la duración de esta etapa que es la más delicada del proceso. Además, en ambos sistemas se pudo tratar el efluente de un digestor de lodos de una planta de tratamiento de aguas residuales alcanzando unas tasas máximas de eliminación de nitrógeno de 1,1 g N L^{-1} d^{-1} a temperatura ambiente, comparables con las máximas obtenidas en el rango de temperaturas óptimo de operación de las bacterias Anammox (superiores a 30 ºC).

4. Transporte de materia en el interior de los gránulos

A diferencia de los sistemas de lodos activos, en el caso de la biomasa granular la actividad de las diferentes poblaciones presentes en los agregados va a depender de la difusión de los sustratos al interior de los mismos. El uso de microelectrodos para la determinación de los perfiles de concentración de nitrito y oxígeno en el interior de los gránulos del sistema CANON permitió comprobar que las condiciones ambientales en el interior de los gránulos eran diferentes a las del medio líquido. El conocimiento de los gradientes de concentración de oxígeno y nitrito en el interior de los gránulos puede usarse para establecer las condiciones óptimas de operación, en términos de concentración de amonio y nitrito en la fase líquida, y diseñar un sistema de control actuando sobre dos variables: la concentración de OD y el tiempo de residencia hidráulico.

El uso de los microelectrodos permitió corroborar la limitación de la concentración de oxígeno así como la estratificación de las diferentes poblaciones bacterianas dentro de los agregados. Las BOA se encontraban en la superficie de los agregados donde consumían el oxígeno que penetraba hasta un máximo de 400 μm en el caso de trabajar con oxígeno puro en el medio líquido, mientras que las Anammox se encontraban localizadas a profundidades entre 400 y 1000 μm para gránulos con un diámetro medio de 5 mm.

5. Modelado de gránulos aerobios

Se desarrolló un modelo capaz de simular tanto las concentraciones de compuestos solubles como de compuestos particulados basándose en una definición de la biomasa que incluyó un perfil de porosidad dentro del gránulo, observado por otros autores de forma experimental, para una representación más realista de las poblaciones de bacterias. El modelo permite determinar no solo perfiles de concentración de sustrato sino también perfiles de concentración de las distintas fracciones particuladas (incluyendo las poblaciones bacterianas) presentes en el gránulo.

6. Aplicación

Con la información obtenida en los trabajos realizados en el ámbito de esta tesis se puede concluir que, el proceso CANON a escala piloto o industrial es viable incluso a temperatura ambiente y que la puesta en marcha óptima consistiría en los siguientes pasos: 1) el desarrollo de una población nitrificante granular, 2) el control de la concentración de oxígeno disuelto para obtener la nitrificación parcial del 50% del amonio a nitrito y 3) la inoculación de biomasa Anammox para acelerar el proceso. Una vez desarrollado el proceso, el

sistema de control consistiría en la monitorización de las concentraciones de amonio y nitrito en el reactor y en la actuación sobre la concentración de oxígeno disuelto y el tiempo de residencia hidráulico.

Conclusións

Preséntanse aquí as conclusións xerais deste traballo de investigación, no que se avaliaron e desenvolveron diferentes poboacións bacterianas en agregados ou gránulos para a eliminación autótrofa de nitróxeno.

1. Granulación de bacterias autótrofas

Dos resultados obtidos conclúese que é posible desenvolver as distintas poboacións bacterianas involucradas na eliminación autótrofa de nitróxeno (oxidantes de amonio, de nitrito e Anammox) en forma de gránulos utilizando reactores secuenciais (SBR: Sequencing batch reactor). Os gránulos oxidantes de amonio obtivéronse a partir de gránulos orixinalmente heterótrofos onde se eliminaba o nitróxeno mediante nitrificación-desnitrificación e nos que se foi retirando progresivamente a fonte de materia orgánica da alimentación. Os gránulos oxidantes de nitrito foron obtidos a partir de gránulos nitrificantes, cambiando a fonte de nitróxeno na alimentación de amonio a nitrito. No caso dos gránulos Anammox, o seu desenvolvemento levouse a cabo a través da adición de cloruro sódico para fomentar a formación dun precipitado que favoreceu a compactación dos gránulos formados. En todos os casos as propiedades de sedimentación dos gránulos (índice volumétrico de lamas, densidade, tamaño de partícula, etc.) foron suficientemente boas para garantir unha óptima retención da biomasa no sistema.

2. Efectos da concentración de osíxeno disolto

O uso de biomasa granular implica a presenza de resistencias á transferencia de materia, o que provoca un gradiente de concentracións no interior do gránulo. No caso dos sistemas aerobios, o transporte de osíxeno disolto (OD) desde a fase líquida cara ao gránulo é xeralmente a etapa limitante e, polo tanto, a que marca a velocidade global do proceso. Isto permite operar o sistema en condicións limitantes de osíxeno malia ter concentracións de OD altas na fase líquida, o que favorece o desenvolvemento de poboacións bacterianas cunha alta afinidade por ese substrato.

Ao alimentar o reactor cun medio autótrofo con amonio como fonte de nitróxeno, a limitación por OD causou que a poboación predominante nos gránulos fose a oxidante de amonio debido á súa maior afinidade polo osíxeno en comparación coa poboación oxidante de nitrito. Baseándose neste principio foi posible a operación estable dun sistema granular nitrificante levando a cabo a nitrificación parcial do 50% de amonio a nitrito a temperatura ambiente. Isto conseguiuse nestas condicións malia que, a temperatura ambiente (ao redor de 20 ºC), as velocidades máximas de crecemento das bacterias oxidantes de nitrito (BON) son superiores ás das bacterias oxidantes de amonio (BOA).

No caso de poboacións nitrito oxidantes, un aumento no fluxo de osíxeno cara ao gránulo causou a evolución das poboacións estrategas da k (*Nitrospira*) cara a estrategas da r (*Nitrobacter*).

3. Operación de sistemas CANON a temperatura ambiente

O feito de ter unha poboación de BOA nos gránulos aerobios que consome o osíxeno disolto do medio para oxidar parcialmente o amonio a nitrito permite a presenza de zonas anóxicas no interior dos gránulos onde poden crecer as bacterias Anammox usando o nitrito e o amonio sobrantes levando a cabo o proceso denominado CANON. No caso do reactor SBR aireado en continuo no que as concentracións de OD eran de entre 2 e 4 mg O_2 L^{-1} promoveuse a formación de gránulos coas poboacións estratificadas, de forma que as

bacterias Anammox colocáronse no interior mentres que as BOA atopábanse nas capas máis externas. Cando a concentración de osíxeno disolto forneceuse mediante un sistema de pulsación, as concentracións de OD no medio foron inferiores (entre 0,3 e 0,5 mg O_2 L^{-1}) e permitiu que as bacterias Anammox creceran fundamentalmente en forma de gránulos mentres que as AOB fixérono en forma de biomasa en suspensión. A posta en marcha realizouse partindo dun sistema de nitrificación parcial no que se inoculou biomasa pobremente enriquecida en bacterias Anammox. Isto permitiu o arranque do proceso CANON nun período dun mes diminuíndo de xeito significativo a duración desta etapa que é a máis delicada do proceso. Ademais, nos dous sistemas púidose tratar o efluente dun dixestor de lamas dunha planta de tratamento de augas residuais alcanzando unhas taxas máximas de eliminación de nitróxeno de 1,1 g N L^{-1} d^{-1} a temperatura ambiente, comparables coas máximas obtidas no rango de temperaturas óptimo de operación das bacterias Anammox (temperaturas superiores a 30 ºC).

4. Transporte de materia no interior dos gránulos

A diferenza dos sistemas de lamas activas, no caso da biomasa granular a actividade das diferentes poboacións presentes nos agregados vai depender da difusión dos substratos ao interior dos mesmos. O uso de microelectrodos para a determinación dos perfís de concentración de nitrito e osíxeno no interior dos gránulos do sistema CANON permitiu comprobar que as condicións ambientais no interior dos gránulos eran diferentes ás do medio líquido. O coñecemento dos gradientes de osíxeno e nitrito no interior dos gránulos pode usarse para establecer as condicións de operación óptimas, en termos de concentración de amonio e nitrito na fase líquida, e deseñar un sistema de control actuando sobre dúas variables: a concentración de osíxeno disolto e o tempo de residencia hidráulico.

O uso dos microelectrodos permitiu corroborar a limitación por concentración de osíxeno así como a estratificación das diferentes poboacións bacterianas dentro dos agregados. As BOA atopábanse na superficie dos gránulos onde consumían o osíxeno que penetraba ata un máximo de 400 μm no caso de traballar con osíxeno puro no medio líquido, mentres que as Anammox atopábanse localizadas a profundidades entre 400 e 1000 μm para gránulos cun diámetro medio de 5 mm.

5. Modelado de gránulos aerobios

Desenvolveuse un modelo capaz de simular tanto as concentracións de compostos solubles como de compostos particulados baseándose nunha definición da biomasa que incluíu un perfil de porosidade dentro do gránulo, observado por outros autores de xeito experimental, para unha representación máis realista das poboacións de bacterias. O modelo permite determinar non só perfís de concentración de substrato senón tamén perfís de concentracións das distintas fraccións particuladas (incluíndo as poboacións bacterianas) no interior do gránulo.

6. Aplicación

Coa información obtida nos traballos realizados no ámbito desta tese pódese concluír que, o proceso CANON a escala piloto ou industrial é viable inclusive a temperatura ambiente e que a posta en marcha óptima do sistema consistiría nos seguintes pasos: 1) o desenvolvemento dunha poboación nitrificante granular, 2) o control da concentración de osíxeno disolto para obter a nitrificación parcial do 50% do amonio a nitrito e 3) a inoculación de biomasa Anammox para acelerar o proceso. Unha vez desenvolvido o proceso, o sistema de control consistiría na monitorización das concentracións de amonio e nitrito no reactor e na actuación sobre a concentración de osíxeno disolto e o tempo de residencia hidráulico.

Conclusions

The main conclusions of this research, which was focused on the development of different bacterial populations in aggregates or granules for autotrophic nitrogen removal, are now presented.

1. Granulation of autotrophic bacteria

From the results obtained it can be concluded that the bacterial populations involved in the different processes of autotrophic nitrogen removal can be developed in granular form using sequencing batch reactors (SBR). Granules enriched in ammonium oxidizing bacteria (AOB) were obtained from heterotrophic granules which were previously performing nitrogen removal by nitrification-denitrifcation before the organic matter in the influent was stepwise removed. Granules enriched in nitrite oxidizing bacteria (NOB) were obtained from nitrifying granules changing the nitrogen source in the feeding from ammonium to nitrite. In the case of Anammox aggregates, their development was performed through the addition of sodium chloride to enhance the formation of precipitates which favoured the compaction of the granules formed. In all the cases the good settling properties of the granules (sludge volume index, density, diameter, etc.) guaranteed optimal biomass retention in the reactor.

2. Effects of dissolved oxygen concentration

By using granular biomass, mass transfer resistances are introduced in the system what causes concentration gradients inside the aggregates. In the case of aerobic granules, the transport of dissolved oxygen (DO) from the bulk liquid to the granule is generally the limiting step, and it determines the global rate of the process. It is therefore possible to operate under oxygen limitation, in spite of working at high DO concentrations in the bulk liquid, to enhance the growth of the bacterial populations with higher oxygen affinity.

When feeding a granular reactor with an autotrophic media with ammonium as nitrogen source, the DO limitation caused the predominance of AOB over NOB. Based on that principle, a granular system carrying out partial nitrification of 50% of ammonium to nitrite at room temperature was established. This objective was reached even working at temperatures (around 20ºC) where the maximal growth rates of NOB are higher than that of AOB.

An experiment performed with NOB revealed that an increase in the flux of oxygen from the bulk liquid to the granule made the bacterial population evolve from a majority of *Nitrospira* (k strategist) to a majority of *Nitrobacter* (r strategist).

3. Operation of CANON systems at room temperature

The AOB developed in the granular biomass consume DO to partially oxidize ammonium to nitrite creating anoxic zones in the inner core of the granules. In this inner core Anammox biomass can grow consuming the available ammonium and nitrite. When working with a SBR continuously aerated with DO concentration in the bulk liquid ranging from 2 to 4 mg O_2 L^{-1}, the formation of granules was promoted. The bacterial populations were stratified, AOB were placed in external layers whereas Anammox bacteria were placed in more internal layers. When working with the SBR with an air pulse device, the DO concentration in the bulk liquid was lower: 0.3-0.5 mg O_2 L^{-1}. In this case, Anammox bacteria grew in the form of granules but AOB grew mainly in suspension.

The start-up of the CANON process was performed starting with a partial nitrification granular system in which an inoculum poorly enriched in Anammox bacteria was introduced. This allowed the start-up of the process in only one month, decreasing significantly the time required to perform this critical stage that. In both systems effluents from anaerobic digesters were treated achieving a maximal nitrogen removal rate of 1.1 g N L^{-1} d^{-1} at room temperature. This value is comparable with the maximal nitrogen removal rates obtained in CANON reactors even working at higher temperatures (more than 30 ºC) closer to the optimum for Anammox bacteria.

4. Mass transfer in the granules

One of the differences between activated sludge and granular systems is that, in the last ones, the activity of the different bacterial populations present depends on the diffusion rate of the substrates from the bulk liquid. Microelectrodes were used to obtain profiles of nitrite and oxygen concentrations into the granules of the CANON system. It was corroborated that the substrates concentrations inside the granules differed from the bulk liquid conditions. The gradients of oxygen and nitrite concentrations into the granules can be used to establish optimal conditions of operation in terms of ammonium and nitrite concentrations and to design a control system with two actuation variables: dissolved oxygen concentration and hydraulic residence time.

Obtained results from microelectrodes corroborated the limitation by DO concentration and also the stratification of the different biomass populations. AOB were placed in the external layers of the granules where the oxygen penetrated until a maximal depth of 400 μm when pure oxygen was applied. Anammox bacteria were placed between 400 μm and 1000 μm of depth in granules with a mean diameter of 5 mm.

5. Modelling of aerobic granules

A model able to simulate the concentrations of the soluble and the particulate compounds present in an aerobic granular SBR was developed. In order to obtain a more realistic model of the particulate fractions, a porosity profile inside the granule, which was already reported by several authors, was defined. The model allowed the determination of not only substrate profiles inside the granule but also the profile of the different particulate fractions (including bacterial populations).

6. Application

With the information obtained from this research it can be concluded that the CANON process can be developed at pilot or industrial scale even at room temperature. An optimized start-up of the process is proposed: 1) development of a granular nitrifying population, 2) the control of the DO concentration in the bulk liquid in order to obtain partial nitrification of 50% of the ammonium to nitrite and 3) the inoculation of Anammox biomass to speed up the process. Once the process started, the control system would consist of the monitoring of ammonium and nitrite concentrations in the bulk liquid and the actuation on the DO concentration and the hydraulic retention time.

List of symbols

1. Acronyms

Anammox	Anaerobic Ammonium Oxidation
AS	Activated Sludge
ASM	Activated Sludge Model
AOB	Ammonia Oxidizing Bacteria
BAS	Biofilm Airlift Suspension Reactor
CANON	Complete autotrophic nitrogen removal
CSTR	Continuous Stirring Tank Reactor
FISH	Fluorescent in situ hybridization
IC	Internal Circulating
I.E.	Inhabitant equivalent
MABR	Membrane Aerated Biofilm Reactor
MBBR	Moving Bed Biofilm Reactor
MBR	Membrane-assisted Bioreactor
NOB	Nitrite Oxidizing Bacteria
OLAND	Oxygen-Limited Autotrophic Nitrification-Denitrification
PAO	Polyphosphate Accumulating Organisms
PHB	Poly-Hydroxy-Butyrate
PCR	Polymerase Chain Reaction
RBC	Rotating Biofilm Contactor
SBR	Sequencing Batch Reactor
SBRP	Air pulsing Sequencing Batch Reactor
SNAP	Single-stage Nitrogen removal using Anammox and Partial nitritation
UASB	Upflow Anaerobic Sludge Blanket
WWTP	Wastewater Treatment Plant

2. Symbols

a	Specific surface of the granules	$m^2\ m^{-3}$
A	Total surface of the granules	m^2
ANR	Nitrogen removal by Anammox bacteria	$g\ N\ L^{-1}\ d^{-1}$
AOR	Ammonium Oxidizing Rate	$g\ N\ L^{-1}\ d^{-1}$
b	Endogenous respiration rate	d^{-1}
COD	Chemical Oxygen Demand	$g\ L^{-1}$

C_S	Concentration of substance S	g L^{-1}
D	Diameter	mm
DBL	Diffusive Boundary Layer	µm
D_S	Diffusivity coefficient of substance S	m^2 d^{-1}
DO	Dissolved Oxygen concentration	g O_2 L^{-1}
FA	Free Ammonia	g N L^{-1}
FNA	Free Nitrous Acid	g N L^{-1}
HRT	Hydraulic Retention Time	d
IC	Inorganic Carbon	g L^{-1}
ISS	Inorganic Suspended Solids	g L^{-1}
J	Flux	g m^{-2} d^{-1} or g d^{-1}
K_S	Half saturation constant of substance S	g L^{-1}
k_C	Liquid-granule mass transfer coefficient	m d^{-1}
k_La	Gas-liquid oxygen transfer coefficient	d^{-1}
n	Number of granules	–
NLR	Nitrogen Loading Rate	g N L^{-1} d^{-1}
NOR	Nitrite Oxidation Rate	g N L^{-1} d^{-1}
OLR	Organic Loading Rate	g COD L^{-1} d^{-1}
Pe	Peclet number	–
R	Radius	mm
Re	Reynolds number	–
SAA	Specific Anammox Activity	g N (g VSS)$^{-1}$ d^{-1}
Sc	Schmidt number	–
Sh	Sherwood number	–
SRT	Solids Retention Time	d
SVI_n	Sludge Volume Index, after n minutes of settling	mL (g VSS)$^{-1}$
T	Temperature	ºC
TOC	Total Organic Carbon	g L^{-1}
TSS	Total Suspended Solids	g L^{-1}
u_{Det}	Detachment rate	m d^{-1}
v_g	Settling velocity of the granules	m h^{-1}
V	Volume	L
VSS	Volatile Suspended Solids	g L^{-1}
X	Biomass concentration	g L^{-1}
Y	Yield coefficient	g COD-X (g COD-S)$^{-1}$

3. Greek symbols

δ	Oxygen penetration depth	µm
δ_H	Effective diffusive boundary layer	µm
ε	Porosity	–
μ	Biomass growth rate	d^{-1}
ρ	Density	$g\ L^{-1}$
v	Cinematic viscosity	$m^2\ d^{-1}$

4. Superindex

A	Ammonia oxidizing bacteria
exp	Experimental
H	Heterotrophic bacteria
inf	Influent
max	Maximal
N	Nitrite oxidizing bacteria
STO	Storage products

5. Subindex

Alk	Alkalinity
HNO2	Free nitrous acid
i	Influent
L	Bulk liquid
m	Mean
max	Maximal
NH3	Free ammonia
NH4	Ammonium
NO2	Nitrite
O2	Oxygen
R	Reactor
s	Surface of the granule
T	Total

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1.1. International journal publications

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