Towards the improvement of start-up and operation of Anammox reactors

Isaac Fernández Rodríguez

PhD Thesis

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Isaac Fernández Rodríguez
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Ramón Méndez Pampín, Catedrático de Ingeniería Química y José Luis Campos Gómez, Profesor Contratado Doctor de Ingeniería Química de la Universidad de Santiago de Compostela,

Informan:

Que la memoria titulada "Towards the improvement of start-up and operation of Anammox reactors", que para optar al grado de Doctor en Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Don Isaac Fernández Rodríguez, 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 a 8 de junio de 2010.

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Esta memoria fue presentada el día 22 de octubre de 2010 en el Salón de Actos de la Escuela Técnica Superior de Ingeniería de la USC ante el Tribunal compuesto por:

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Objetivos y resumen

La presente tesis doctoral se enmarca en la depuración biológica de aguas residuales y específicamente en la eliminación de compuestos nitrogenados. Hay tres principales problemas ambientales que pueden ser producidos por la presencia de contaminantes nitrogenados en los sistemas acuáticos: pueden disminuir el pH de los ecosistemas de agua dulce cuya alcalinidad sea relativamente baja, llevando a la acidificación de esas masas de agua; pueden estimular o incrementar el desarrollo y proliferación de los organismos fotosintéticos, resultando en la eutrofización de los sistemas acuáticos; y pueden también causar toxicidad a la vida acuática, con un incremento de la mortalidad y problemas reproductivos. Además, la contaminación por compuestos de nitrógeno de las aguas subterráneas y superficiales puede provocar efectos adversos en la salud humana, como toxicidad aguda, carcinogénesis, mutagénesis y alergias.

El nitrógeno se suele encontrar en las aguas residuales mayoritariamente en forma de amonio. Los procesos biológicos convencionales usados para su eliminación en las plantas de tratamiento de aguas residuales se basan en los procesos de nitrificación y desnitrificación. En la nitrificación el amonio se oxida primero a nitrito y después a nitrato, por medio de las bacterias oxidantes de amonio y las bacterias oxidantes de nitrito, respectivamente. El consumo total de oxígeno necesario para convertir el amonio a nitrato es alrededor de 4,2-4,5 g O_2 (g $N\text{-}NH_4^+)^{-1}$. Además, la nitrificación causa un consumo de alcalinidad de aproximadamente 7,1 g $CaCO_3$ (g $N\text{-}NH_4^+)^{-1}$. Esta alcalinidad puede estar ya presente en el agua residual a tratar o añadirse químicamente. A continuación, el nitrato y/o nitrito formados se reducen a nitrógeno gaseoso por medio de bacterias desnitrificantes que usan materia orgánica como donador de electrones.

Sin embargo existen aguas residuales cuya cantidad de materia orgánica biodegradable no es suficiente para llevar a cabo la desnitrificación. En este caso es necesario añadir una fuente externa de carbono que permita completar la desnitrificación, lo que tiene asociado un coste económico. Algunas fuentes externas de carbono típicas son alcoholes de cadena corta (como metanol y etanol), acetato y glucosa. Habitualmente el metanol es la fuente de carbono más barata disponible, por lo que es el compuesto más utilizado. La cantidad de materia orgánica que se necesita por unidad de masa de nitrógeno eliminado es de alrededor de 3,7 g DQO $(g\ N)^{-1}$ cuando se emplea metanol como fuente externa de carbono.

Una alternativa relativamente reciente para la eliminación de nitrógeno en aguas con una baja relación DQO/N son las tecnologías basadas en la oxidación anaerobia de amonio (Anammox). Este proceso lo realizan bacterias autótrofas que combinan amonio y nitrito en condiciones anóxicas para dar lugar a nitrógeno gaseoso y una pequeña cantidad de nitrato. Dado que habitualmente el nitrito no está presente en las aguas residuales, es necesario oxidar en torno al 50% del amonio a nitrito (nitrificación parcial).

La nitrificación parcial y el proceso Anammox pueden llevarse a cabo en dos unidades independientes. En este caso el primer reactor se opera en condiciones aerobias para convertir aproximadamente la mitad del amonio presente en el influente en nitrito. En el segundo reactor se lleva a cabo la desnitrificación autotrófica (Anammox) en condiciones anóxicas.

La nitrificación parcial y el proceso Anammox pueden también realizarse conjuntamente en una sola unidad. Esta tecnología ha recibido diversos nombres como CANON (Completely Autotrophic Nitrogen removal Over Nitrite; eliminación de nitrógeno completamente autotrófica por la vía del nitrito); OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification; nitrificación-desnitrificación autotrófica en condiciones de limitación de oxígeno) y deamonificación. La principal diferencia entre todas estas

tecnologías es que el proceso CANON emplea biomasa en suspensión desarrollándose conjuntamente las diversas especies bacterianas en un medio de reacción bien mezclado, mientras que los procesos OLAND y de deamonificación emplean biomasa en forma de biopelícula, que crece sobre biodiscos o sobre soportes de plástico, como los anillos Kaldnes. La concentración de oxígeno disuelto es la principal variable de operación que debe controlarse a fin de conseguir una operación estable del sistema. Las limitaciones difusionales permiten que el oxígeno sea consumido completamente en la zona externa del gránulo o de la biopelícula, con lo que la zona interna se mantiene anóxica. Por lo tanto, la nitrificación parcial ocurre en la parte externa mientras que las bacterias Anammox crecerán en las capas más internas.

En cualquier caso e independientemente de la configuración de los reactores, tanto la nitrificación como el proceso Anammox son autótrofos por lo que no se necesita la presencia de materia orgánica biodegradable para llevar a cabo la eliminación de nitrógeno. Además las necesidades de oxígeno son alrededor del 40% inferiores cuando se comparan con las de la nitrificación/desnitrificación convencional porque sólo se necesita oxidar el 50% del amonio a nitrito. La producción de lodo también es muy inferior debido a la reducida productividad de biomasa de los organismos autótrofos. Por lo tanto, el proceso de nitrificación parcial/Anammox puede considerarse más barato y más sostenible que la nitrificación/desnitrificación convencional.

A pesar de las ventajas del proceso Anammox, todavía existen algunos aspectos importantes que deben ser estudiados a fin de mejorar sus posibilidades de implementación a escala industrial. Algunos de esos aspectos son el objeto de la investigación desarrollada en la presente tesis doctoral. Específicamente, la mejora de la retención de la biomasa en el interior de los reactores, la evaluación de los efectos de los posibles inhibidores del proceso y la aplicación del proceso en el rango mesofílico de temperatura.

A continuación se resumen los contenidos principales de cada uno de los capítulos de la tesis:

En el **Capítulo 1** se realiza una revisión bibliográfica de la problemática de la eliminación de nitrógeno de las aguas residuales. También se lleva a cabo una descripción de las principales características del proceso Anammox y sus condiciones de operación. Seguidamente se incluyen análisis económicos de la eliminación de nitrógeno de las aguas residuales mediante diferentes tecnologías. Y para finalizar se realiza una discusión acerca de las perspectivas futuras de la eliminación de nitrógeno mediante el proceso Anammox.

En el **Capítulo 2** se detallan los materiales y métodos experimentales utilizados en la realización de los experimentos de los capítulos posteriores de esta tesis doctoral. Primero se explican los métodos empleados para analizar las propiedades de la fase líquida. A continuación de éstos, se incluyen las técnicas para la caracterización física y microbiológica de la biomasa. Finalmente, se detalla la composición del medio mineral Anammox. Este medio se usó como base para preparar la alimentación sintética de los reactores.

En el **Capítulo 3** se estudia la inhibición del proceso Anammox causada por sus sustratos (nitrito y amonio). Se asumió que las responsables de la inhibición eran las especies no ionizadas de los sustratos (amonio libre y ácido nitroso libre). Una ventaja de usar niveles mínimos de inhibición en términos de estos compuestos no ionizados es que la aplicación al control de diferentes sistemas Anammox operando en diferentes condiciones puede ser más sencilla.

En una primera parte se determinaron los efectos inhibitorios a corto plazo de ambos sustratos mediante ensayos de actividad. Se utilizaron dos tipos diferentes de biomasa Anammox (biopelícula sobre soportes inorgánicos y lodo floculento). Se estimó un valor de IC50 (concentración

que causa un 50% de inhibición) para el amonio libre de 35 mg $N\text{-}NH_3$ L^{-1}, sin diferencias significativas entre los dos tipos de biomasa usados. En el caso del ácido nitroso, el valor de IC50 para la biomasa en biopelícula fue de 11 µg $N\text{-}HNO_2$ L^{-1}. Sin embargo, la biomasa floculenta fue mucho menos resistente y su actividad específica fue inferior al 30% en presencia de sólo 4,4 µg $N\text{-}HNO_2$ L^{-1}. Seguidamente, se emplearon reactores SBR inoculados con biomasa en biopelícula para estudiar los efectos de la exposición a largo plazo. Se encontró que dichos efectos son más importantes que los observados durante la exposición a corto plazo. De ese modo, en presencia de 35-40 mg $N\text{-}NH_3$ L^{-1} y de 1,5 µg $N\text{-}HNO_2$ L^{-1} no se pudo conseguir la operación estable del reactor.

El **Capítulo 4** se dedica al estudio de los efectos de dos antibióticos de amplio espectro, como son el cloranfenicol y el hidrocloruro de tetraciclina, sobre el proceso Anammox. Este estudio tiene como finalidad conocer si es posible tratar con el proceso Anammox aguas procedentes de la digestión anaerobia de purines, las cuales se espera que contengan concentraciones de antibióticos relativamente elevadas. Inicialmente se determinaron los posibles efectos a corto plazo mediante ensayos de actividad y de biotoxicidad. Se encontró que ambos antibióticos producen un potente efecto inhibitorio y además, el hidrocloruro de tetraciclina causó un efecto de desactivación de la biomasa. Los ensayos de actividad Anammox y de biotoxicidad dieron similares valores de IC50 para el cloranfenicol, alrededor de 400 mg L^{-1}. Sin embargo, en el caso del hidrocloruro de tetraciclina, el IC50 calculado a partir de los ensayos de actividad fue de alrededor de 220 mg L^{-1}, muy superior a 94 y 42 mg L^{-1}, que fueron los valores de IC50 después de 5 y 15 min obtenidos con los ensayos de biotoxicidad. La diferencia entre los resultados de biotoxicidad a 5 y 15 min confirmaría el incremento de los efectos tóxicos de la tetraciclina con el tiempo.

A continuación se investigaron los efectos a largo plazo añadiendo cada uno de los antibióticos a la alimentación de un reactor SBR Anammox. Se

observó que 20 mg L^{-1} de cloranfenicol producían una disminución del 80% en la actividad Anammox. Efectos similares se observaron operando el reactor en presencia de 50 mg L^{-1} de hidrocloruro de tetraciclina. A pesar de la pérdida de actividad, los antibióticos no causaron cambios en las propiedades físicas de la biomasa, lo que permitió una buena retención de la biomasa. Puesto que la presencia de estos antibióticos fue perjudicial para la operación del proceso Anammox, en el capítulo también se discuten las posibles alternativas para su degradación previa.

El proceso Anammox se ha operado en la gran mayoría de los casos, tanto en reactores de laboratorio como de tamaño industrial, para tratar aguas residuales a temperaturas próximas a 30 ºC. Estas aguas suelen proceder de tratamientos anaerobios operados en el rango mesófilo. En el **Capítulo 5** se estudia la posibilidad de operar el proceso a temperaturas inferiores, lo cual permitiría su aplicación a aguas residuales de diferentes procedencias. En una primera etapa se estudiaron los efectos de la temperatura a corto plazo por medio de ensayos de actividad. Se encontró una tendencia de tipo Arrhenius con una energía de activación de 63 kJ mol^{-1} y el óptimo en el intervalo 35-40 ºC. A continuación se fue disminuyendo gradualmente la temperatura de operación de un reactor SBR Anammox con el fin de detectar una posible adaptación de la biomasa. El sistema fue operado eficientemente a 18 ºC, observando que la resistencia de la biomasa sometida en continuo a bajas temperaturas era mayor que la encontrada durante los ensayos de actividad. Sin embargo, cuando la temperatura de operación fue de 15 ºC, se empezó a acumular nitrito (sustrato limitante) en el medio de reacción y el sistema perdió su estabilidad. Finalmente, se estudió el tratamiento del sobrenadante de un digestor anaerobio a 20 ºC en un sistema con dos reactores SBR en serie, que llevaban a cabo la nitrificación parcial y el proceso Anammox. La carga nitrogenada global eliminada por el sistema fue de 0,08 g N $(L\ d)^{-1}$.

Después de investigar de forma independiente los efectos de los sustratos y la temperatura sobre el proceso Anammox, en el **Capítulo 6** se emplea una herramienta estadística (modelos de superficie de respuesta) para evaluar la influencia combinada de distintas variables. En este caso se tomó la biomasa procedente de un reactor de deamonificación (proceso Anammox de una etapa en biopelícula) y se usó la actividad Anammox específica como variable de respuesta. Se seleccionaron como variables controladas la temperatura, el pH, la concentración de amonio, la concentración de nitrógeno total y la relación entre amonio libre y ácido nitroso. Se observó que los parámetros más importantes a la hora de optimizar la actividad Anammox eran la temperatura, el valor de pH y también la relación entre amonio libre y ácido nitroso. Además se encontraron sus intervalos óptimos. En función de esta información se propuso una estrategia de control para un reactor de deamonificación.

Debido a la lenta velocidad de crecimiento de la biomasa Anammox y a su reducida productividad celular, es importante mantener una buena retención de la biomasa en el interior de los reactores. Esto es especialmente importante durante la puesta en marcha cuando se parte de una pequeña cantidad de inóculo. En este sentido, una de las alternativas que se han propuesto para la mejora de la retención de la biomasa Anammox es la formación de biopelículas. Los dos últimos capítulos de esta tesis se centraron en esta tecnología. En el **Capítulo 7** se estudia la influencia del estrés mecánico y la salinidad en la formación de biopelículas Anammox. El desarrollo de las biopelículas se siguió mediante un sensor basado en las propiedades de vibración superficial. En general, se observó que la biomasa Anammox tiene una buena capacidad para formar biopelículas, durando la fase de adhesión inicial de la biomasa al soporte de 5-7 días para los tres diferentes caudales empleados (25,2; 8,4; 7,3 L h^{-1}) que correspondieron a la aplicación de flujos con Reynolds 188, 63 y 54. La estabilidad de la biopelícula fue mayor cuando se formó bajo condiciones de estrés mecánico más alto. Además se observó que la presencia de sales (NaCl, $CaCl_2$) favorecía la

formación de la biopelícula, debido a la reducción de las fuerzas de repulsión electrostática. Los efectos del $CaCl_2$ fueron mayores que los causados por el NaCl probablemente debido a la formación de puentes catiónicos divalentes. En los dos casos se observó la incorporación de compuestos inorgánicos a la biopelícula.

Finalmente, en el **Capítulo 8** se estudia la puesta en marcha y operación de un sistema Anammox de biopelícula. Se eligió la zeolita natural como soporte para la biomasa, debido a su capacidad para adsorber amonio, que es uno de los sustratos consumidos por los organismos Anammox. De este modo, la biomasa en forma de biopelícula podría tener un mejor acceso al NH_4^+ y se fomentaría la formación de dicha biopelícula. Se observó que para promover la formación y crecimiento de la biopelícula Anammox era crucial mantener baja la concentración de amonio en el medio de reacción, de modo que la mayor parte de dicho sustrato se encontrara adsorbido sobre las zeolitas.

Una vez que se logró que se desarrollara la biopelícula, la retención de biomasa que se alcanzó en el sistema fue muy buena, con concentraciones de sólidos en suspensión volátiles en el efluente inferiores a 3 mg SSV L^{-1}. Como consecuencia, la concentración de biomasa en el reactor se incrementó significativamente. Además, se logró una mejora en la actividad específica de la biomasa, alcanzando valores de hasta 0,5 g N (g SSV $d)^{-1}$.

La elevada densidad de las partículas de zeolita implicó la necesidad de aplicar una potencia de agitación relativamente elevada al medio de reacción, a fin de mantener dichas partículas en suspensión. Durante la puesta en marcha, la agitación aplicada podría producir una abrasión causada por partículas de zeolita no cubiertas, lo que tendría efectos perjudiciales para la biopelícula. Además, el estrés mecánico en si mismo podría causar un descenso en la actividad específica de la biomasa. Una vez que todas o la gran mayoría de las partículas se encontraron

completamente cubiertas por biomasa, este problema se minimizó debido al descenso en la densidad global de las partículas y a la menor potencia de agitación necesaria. Además las partículas cubiertas por biopelícula tienen menor capacidad abrasiva.

Debido a que el crecimiento de la biopelícula se consiguió cuando la concentración de amonio en el líquido se mantuvo relativamente baja, un reactor operando con esta tecnología debe alimentarse con una relación de sustratos próxima a la estequiométrica. Para conseguir esta relación el reactor de nitrificación parcial que produce el nitrito debe controlarse de forma cuidadosa. Esto puede resultar complicado durante los períodos de puesta en marcha. Un inconveniente adicional podría ser el hecho de que, teniendo en cuenta que el proceso Anammox puede ser inhibido por nitrito, tal como se demuestra en el Capítulo 3, la operación con un exceso significativo de amonio podría considerarse más segura, especialmente cuando las concentraciones en el influente no son muy estables. Sin embargo, debido a que una cantidad de amonio se encontrará adsorbida en el soporte sólido durante la operación, el sistema podrá actuar como un "tampón de amonio" y mitigar en cierta medida las sobrecargas por nitrito. De hecho, esta puede ser una de las principales ventajas del uso de las zeolitas como soporte de la biomasa, cuando se comparan con otras tecnologías Anammox de biopelícula (como los anillos Kaldnes), puesto que las zeolitas son una especie de "soporte activo" mientras que el plástico, vidrio y otros tipos de materiales no tienen esta capacidad.

Con los trabajos realizados y expuestos en esta tesis doctoral se ha profundizado en el conocimiento de algunos de los aspectos clave del proceso Anammox como son su puesta en marcha y las condiciones de operación para mantener la estabilidad del sistema. Por lo tanto se considera que el conjunto de conocimientos obtenidos, que fueron resumidos a lo largo de la presente sección, facilitará la implantación industrial de dicho proceso para el tratamiento de diferentes aguas residuales.

Obxectivos e resumo

A presente tese encádrase na depuración biolóxica de augas residuais e, máis especificamente, na eliminación de compostos nitroxenados. Hai tres principais problemas causados pola contaminación por nitróxeno nos sistemas acuáticos: pode facer baixar o pH dos sistemas acuáticos de auga doce que teñan a alcalinidade relativamente baixa, provocando a acidificación desas augas; pode estimular ou incrementar o desenvolvemento e proliferación dos organismos fotosintéticos, resultando na eutrofización dos sistemas acuáticos; e tamén pode causar toxicidade directa sobre a vida acuática, producindo un incremento na mortalidade e nos problemas reprodutivos. Ademais, a contaminación nitroxenada das augas superficiais e subterráneas pode producir efectos adversos para a saúde humana como toxicidade aguda, cancro, mutacións e alerxias.

O nitróxeno aparece nas augas residuais maioritariamente en forma de amonio. Os procesos biolóxicos convencionais empregados para a súa eliminación nas plantas de tratamento de augas residuais están baseados nos procesos de nitrificación e desnitrificación. Na nitrificación o amonio é oxidado primeiro a nitrito e despois a nitrato, por medio das bacterias oxidantes de amonio e oxidantes de nitrito, respectivamente. O consumo total de osíxeno que se necesita para converter o amonio a nitrato é arredor de 4,2-4,5 g O_2 (g $N\text{-}NH_4^+)^{-1}$. Ademais, a nitrificación causa un consumo da alcalinidade do medio e son necesarios 7,1 g $CaCO_3$ (g $N\text{-}NH_4^+)^{-1}$. Esta alcalinidade pode estar xa presente na auga residual a tratar ou ben ser engadida quimicamente. Seguidamente, o nitrato e/ou nitrito formados redúcense a nitróxeno gasoso por medio de bacterias desnitrificantes que empregan materia orgánica como doador de electróns. Sen embargo existen augas residuais con concentracións de materia orgánica biodegradable que non son dabondo para levar a cabo a

desnitrificación. Neses casos é preciso engadir una fonte externa de carbono que permita completar a desnitrificación, o que ten asociado un custo económico. Algunhas fontes externas de carbono típicas son alcois de cadea curta (por exemplo metanol ou etanol), acetato e glicosa. Habitualmente, o metanol é a fonte de carbono dispoñible de menor prezo, polo tanto é o composto máis utilizado. A cantidade de materia orgánica que se precisa por unidade de masa de nitróxeno eliminado é de arredor de 3,7 g DQO $(g\ N)^{-1}$ cando se emprega metanol como fonte externa de carbono.

Unha alternativa relativamente nova para a eliminación de nitróxeno en augas cunha baixa relación DQO/N son as tecnoloxías baseadas na oxidación anaerobia de amonio (Anammox). Este proceso realízano bacterias autótrofas que combinan amonio e nitrito en condicións anóxicas para dar lugar a nitróxeno gasoso e unha pequena cantidade de nitrato. Posto que habitualmente o nitrito non se atopa presente nas augas residuais, é necesario dispoñer dunha unidade nitrificante previa onde se oxide arredor do 50% do amonio a nitrito (nitrificación parcial).

A nitrificación parcial e o proceso Anammox poden levarse a cabo en dúas unidades diferentes. O primeiro dos reactores é operado en condicións aerobias para converter aproximadamente a metade do amonio do influente a nitrito. No segundo reactor lévase a cabo o proceso Anammox (desnitrificación autotrófica en condicións anóxicas).

A nitrificación parcial e mailo proceso Anammox tamén poden realizarse conxuntamente nunha soa unidade. Este tipo de tecnoloxía pode recibir diferentes nomes: CANON (Completely Autotrophic Nitrogen removal Over Nitrite; eliminación completamente autotrófica de nitróxeno pola vía do nitrito); OLAND (Oxygen-Limited Autrotrophic Nitrification-Denitrificaion; nitrificación-desnitrificación autotrófica en condicións de limitación de osíxeno) e deamonificación. A principal diferenza entre eles é que o proceso CANON emprega biomasa en suspensión con diferentes especies

bacterianas que se desenvolven conxuntamente nun medio de reacción ben mesturado. Pola contra, os procesos OLAND e de deamonificación son procesos de biopelícula, polo que a biomasa medra sobre biodiscos ou en soportes de plástico, como os aneis Kaldnes. A concentración de osíxeno no medio líquido é a principal variable de operación que debe controlarse para conseguir a operación estable do sistema. As limitacións difusionais permiten que o osíxeno se consuma na parte externa do gránulo ou da biopelícula, co cal a parte interior se mantén anóxica. Desta maneira, a nitrificación parcial lévase a cabo na parte externa e as bacterias Anammox medran nas capas máis internas.

De calquera xeito, e independentemente da configuración dos reactores, tanto a nitrificación como o proceso Anammox son autótrofos, polo que non é precisa a presenza de materia orgánica para levar a cabo a eliminación de nitróxeno. Ademais, os requirimentos de osíxeno son arredor dun 40% inferiores comparados coa nitrificación/desnitrificación convencional porque só se precisa oxidar o 50% do amonio a nitrito. A produción de lamas é tamén moi inferior debido á baixa produtividade de biomasa dos organismos autotróficos. Polo tanto, o proceso de nitrificación parcial/Anammox pode ser considerado máis barato e máis sostible que a nitrificación/desnitrificación convencional.

A pesar das vantaxes do proceso Anammox, aínda hai varios aspectos que deben ser investigados para mellorar as posibilidades de implantación a escala industrial. Algúns deses aspectos son o obxecto das investigacións desenvolvidas na presente tese doutoral. Especificamente, a mellora da retención da biomasa no interior dos sistemas de reacción, a avaliación dos efectos dos posíbeis inhibidores do proceso e maila aplicación do proceso Anammox no rango mesofílico de temperatura.

Seguidamente resúmense os contidos principais de cada un dos capítulos desta tese:

No **Capítulo 1** lévase a cabo unha revisión bibliográfica da problemática da eliminación de nitróxeno das augas residuais. Tamén se fai unha descrición das principais características do proceso Anammox e as súas condicións de operación. A continuación inclúense análises económicas da eliminación de nitróxeno das augas residuais mediante diferentes tecnoloxías. Para finalizar realizase unha discusión sobre as perspectivas de futuro da eliminación de nitróxeno mediante o proceso Anammox.

No **Capítulo 2** detállanse os materiais e métodos experimentais empregados na realización dos traballos dos capítulos posteriores desta tese de doutoramento. Primeiro explícanse os métodos empregados para analizar as propiedades da fase líquida. Seguidamente, inclúense as técnicas para a caracterización física e microbiolóxica da biomasa. Finalmente, detállase a composición do medio mineral Anammox. Este foi o que se empregou como base para preparar os medios sintéticos de alimentación dos reactores.

No **Capítulo 3** estúdase a inhibición do proceso Anammox causada polos substratos (nitrito e amonio). Considerouse que as especies non ionizadas dos substratos (amonio libre e ácido nitroso libre) eran as responsábeis da inhibición do proceso Anammox. Unha vantaxe de empregar concentracións mínimas de inhibición expresadas en termos de compostos non ionizados é que a aplicación para o control de diferentes sistemas Anammox operados en distintas condicións pode ser máis sinxela.

Nunha primeira parte determináronse os efectos inhibitorios a curto prazo de ámbolos dous substratos por medio de ensaios de actividade. Empregáronse dous tipos diferentes de biomasa Anammox, biopelícula sobre soporte inorgánico e biomasa floculenta. Estimouse un valor de IC50 para o amonio libre (concentración que causa un 50% de inhibición) de 35 mg $N\text{-}NH_3$ L^{-1}, sen diferenzas significativas entre os dous tipos de biomasa empregados. No caso do ácido nitroso, o valor de IC50 para a

biomasa en forma de biopelícula foi de 11 µg $N\text{-}HNO_2$ L^{-1}. Sen embargo, a biomasa floculenta foi moito menos resistente a este composto e a súa actividade específica descendeu por debaixo do 30% en presenza de só 4,4 µg $N\text{-}HNO_2$ L^{-1}. A continuación empregáronse reactores SBR inoculados coa biomasa en forma de biopelícula para estudar os efectos da exposición a longo prazo. Atopouse que ditos efectos son máis importantes que os observados na exposición a curto prazo. Dese xeito a operación do reactor foi totalmente inestable na presenza de 35-40 mg $N\text{-}NH_3$ L^{-1} e de 1,5 µg $N\text{-}HNO_2$ L^{-1}.

O **Capítulo 4** dedícase ó estudo dos efectos de dous antibióticos de amplo espectro, como son o cloranfenicol e o hidrocloruro de tetraciclina, sobre o proceso Anammox. Este estudo ten como obxectivo coñecer se é posíbel o tratamento mediante o proceso Anammox de augas procedentes da dixestión anaerobia de xurros. Pódese esperar que estas augas conteñan concentracións de antibióticos relativamente elevadas. Inicialmente determináronse os efectos a curto prazo mediante ensaios de actividade e biotoxicidade. Atopouse que ámbolos dous antibióticos tiñan un potente efecto inhibitorio e ademais o hidrocloruro de tetraciclina causou un efecto de perda de actividade da biomasa co tempo. Os ensaios de actividade Anammox e de biotoxicidade deron valores similares de IC50 para o cloranfenicol, arredor dos 400 mg L^{-1}. Sen embargo, no caso do hidrocloruro de tetraciclina, o valor de IC50 calculado a partir dos ensaios de actividade foi arredor de 220 mg L^{-1}, moito maior que 94 e 42 mg L^{-1}, que foron os valores de IC50 a 5 e 15 min obtidos por medio dos tests de biotoxicidade. A diferenza entre os resultados destes tests de biotoxicidade a 5 e 15 min confirmaría o incremento dos efectos tóxicos causados pola tetraciclina ó longo do tempo.

Seguidamente investigáronse os efectos a longo prazo, engadindo cada un dos antibióticos á alimentación dun reactor SBR Anammox. Observouse que 20 mg L^{-1} de cloranfenicol producían unha diminución do 80% da actividade Anammox. Efectos semellantes ocorreron cando se

operou o reactor na presenza de 50 mg L^{-1} de hidrocloruro de tetraciclina. A pesar da perda de actividade, ningún dos dous antibióticos causou cambios nas propiedades físicas da biomasa, o cal permitiu manter a boa retención da mesma no medio de reacción. Tendo en conta que a presenza dos antibióticos foi prexudicial para a operación do proceso Anammox, neste capítulo tamén se discuten as posíbeis alternativas para a súa degradación previa.

O proceso Anammox foi operado na maioría dos casos, tanto con reactores de laboratorio como de tamaño industrial, para tratar augas residuais a temperaturas próximas a 30 ºC. Estas augas habitualmente proceden de tratamentos anaerobios que operan no rango mesófilo. No **Capítulo 5** estúdase a posibilidade de operar o proceso a temperaturas inferiores, o cal permitiría a súa aplicación a augas residuais de diferentes procedencias. Nunha primeira etapa estudáronse os efectos da temperatura a curto prazo por medio de ensaios de actividade. Atopouse unha tendencia de tipo Arrhenius cunha enerxía de activación de 63 kJ mol^{-1} e unha actividade óptima no intervalo 35-40 ºC. Seguidamente foise diminuíndo gradualmente a temperatura de operación dun reactor SBR Anammox co obxectivo de detectar unha hipotética adaptación da biomasa. O sistema foi operado de forma eficiente a 18 ºC, observando que a resistencia da biomasa sometida en continuo a baixas temperaturas era maior que a atopada previamente nos ensaios de actividade. Sen embargo, cando a temperatura de operación foi de 15 ºC, empezou a acumularse nitrito (substrato limitante) no medio de reacción e o sistema perdeu a súa estabilidade. Finalmente, estudouse o tratamento do efluente dun dixestor anaerobio a 20 ºC nun sistema de dous reactores SBR en serie, que levaban a cabo a nitrificación parcial e o proceso Anammox. A carga nitroxenada global eliminada polo sistema foi de 0,08 g N $(L\ d)^{-1}$.

Despois de investigar separadamente os efectos dos substratos e maila temperatura sobre o proceso Anammox, no **Capítulo 6** emprégase unha

ferramenta estatística (modelos de superficie de resposta) para avaliar a influencia combinada de distintas variables. Neste caso tomouse a biomasa procedente dun reactor de deamonificación (proceso Anammox dunha etapa en biopelícula) e empregouse a actividade Anammox específica como variable de resposta. As variables controladas foron a temperatura, o pH, a concentración de amonio, a concentración de nitróxeno total e a relación entre amonio libre e ácido nitroso libre. Observouse que as variables máis importantes para a optimización da actividade Anammox eran a temperatura, o valor de pH e tamén a relación entre amonio libre e ácido nitroso. Ademais atopáronse os intervalos óptimos desas variables. En función desta información propúxose unha estratexia de control para un reactor de deamonificación.

Posto que a biomasa Anammox ten unha lenta velocidade de crecemento e unha reducida produtividade celular, é importante manter unha boa retención da biomasa no interior dos reactores. Trátase dun aspecto especialmente importante durante a posta en marcha cando se parte dunha pequena cantidade de inóculo. Deste xeito, unha das alternativas que se propuxeron para a mellora da retención da biomasa Anammox é a formación de biopelículas. Os dous últimos capítulos desta tese centráronse nesta tecnoloxía. No **Capítulo 7** estúdase a influencia do estrés mecánico e a salinidade na formación de biopelículas Anammox. O desenvolvemento das biopelículas seguiuse cun sensor baseado nas propiedades de vibración superficial. En xeral, observouse que a biomasa Anammox ten unha boa capacidade para formar biopelículas, durando a fase de adhesión inicial da biomasa ao soporte de 5-7 días para os tres diferentes caudais empregados (25,2; 8,4; 7,3 L h^{-1}) que corresponderon á aplicación de fluxos con Reynolds 188, 63 e 54. A estabilidade da biopelícula foi maior cando se formou en condicións de estrés mecánico máis elevado. Ademais observouse que a presenza de sales (NaCl, $CaCl_2$) favorecía a formación da biopelícula debido á redución das forzas de repulsión electrostáticas. Os efectos causados polo $CaCl_2$ foron maiores que os producidos polo NaCl, probablemente pola formación de pontes

catiónicas divalentes grazas á presenza do ión calcio. A incorporación de compostos inorgánicos na biopelícula observouse nos dous casos.

Finalmente, no **Capítulo 8** estúdase a posta en marcha e operación dun sistema Anammox de biopelícula. Elixiuse unha zeolita natural como soporte para a biomasa, debido á súa capacidade para adsorber amonio, que é un dos substratos consumidos polos organismos Anammox. Deste xeito, a biomasa formando parte da biopelícula podería ter un mellor acceso ó NH_4^+ e fomentaríase a formación de dita biopelícula. Observouse que para promover a formación e crecemento da biopelícula Anamox era moi importante manter baixa a concentración de amonio no medio de reacción, de forma que a maior parte de dito substrato se atopara adsorbido sobre as zeolitas.

Unha vez que a biopelícula se desenvolveu, a retención de biomasa que se acadou no sistema foi moi boa, con concentracións de sólidos en suspensión volátiles no efluente inferiores a 3 mg SSV L^{-1}. Como consecuencia, a concentración de biomasa no interior do reactor incrementouse de forma significativa. Ademais lograrouse unha mellora da actividade específica da biomasa, acadando valores de ata 0,5 g N (g SSV d)$^{-1}$.

A elevada densidade das partículas de zeolita implicou a necesidade de aplicar unha potencia de axitación relativamente elevada ó medio de reacción, a fin de manter ditas partículas en suspensión. Durante a posta en marcha, a axitación aplicada podería producir unha abrasión causada polas partículas de zeolita non cubertas, o cal tería efectos prexudiciais para a biopelícula. Ademais, o estrés mecánico en si mesmo podería causar un descenso na actividade específica da biomasa. Unha vez que todas ou a gran maioría das partículas se atoparon completamente cubertas por biomasa, este problema foi minimizado debido ó descenso na densidade global das partículas e á menor potencia de axitación

necesaria. Ademais, as partículas cubertas por biopelícula teñen menor capacidade abrasiva.

Debido a que o crecemento da biopelícula se acadou cando a concentración de amonio no reactor se mantivo relativamente baixa, un reactor operando con esta tecnoloxía debe alimentarse cunha relación de substratos próxima á estequiométrica. Para acadar esta relación, o reactor de nitrificación parcial que produce o nitrito ten que controlarse con coidado. Isto pode resultar complicado durante os períodos de posta en marcha. Un inconveniente adicional podería ser o feito de que, tendo en conta que o proceso Anammox pode ser inhibido por nitrito, tal como se demostra no Capítulo 3, a operación cun exceso significativo de amonio pode considerarse máis segura, especialmente cando as concentracións no influente non son moi estables. Sen embargo, debido a que unha cantidade de amonio vai estar adsorbida no soporte sólido durante a operación, o sistema poderá actuar como unha especie de "tampón de amonio" e mitigar en certa medida as sobrecargas por nitrito. De feito, esta pode ser unha das principais vantaxes do uso das zeolitas como soporte para a biomasa, cando se comparan con outras tecnoloxías Anammox de biopelícula (como os aneis Kaldnes), posto que as zeolitas son unha especie de "soporte activo", mentres que o plástico, vidro e outros tipos de materiais non teñen esa capacidade.

Cos traballos levados a cabo e expostos na presente tese doutoral profundouse no coñecemento dalgúns dos aspectos clave do proceso Anammox como son a súa posta en marcha e as condicións de operación para manter a estabilidade do sistema. Polo tanto considérase que o conxunto de coñecementos obtidos, que foron resumidos ó longo da presente sección, facilitará a implantación industrial de dito proceso para o tratamento de diferentes tipos de augas residuais.

Objectives and summary

This doctoral thesis is focused on biological treatment of wastewater and, specifically, on nitrogen removal. There are three main environmental problems caused by nitrogen pollution in aquatic ecosystems: it can decrease the pH of freshwater bodies without much alkalinity, leading to their acidification; it can stimulate or enhance the development and proliferation of photosynthetic organisms, resulting in eutrophication of aquatic ecosystems; and it can cause direct toxicity in aquatic life, leading to increased mortality and reproductive problems. In addition, nitrogen pollution of ground and surface waters can induce adverse effects on human health, like acute toxicity, carcinogenesis, mutagenesis and allergies.

Nitrogen is usually present in wastewater as ammonium. Biological conventional processes for nitrogen removal in wastewater treatment plants are based on nitrification and denitrification. Nitrification is the oxidation of ammonium firstly to nitrite and then to nitrate, by means of ammonium oxidizing bacteria and nitrite oxidizing bacteria, respectively. Total consumption of oxygen in order to convert ammonium into nitrate is about 4.2-4.5 g O_2 (g NH_4^+-N)$^{-1}$. Furthermore, nitrification causes alkalinity consumption and 7.1 g $CaCO_3$ (g NH_4^+-N)$^{-1}$ are necessary. This alkalinity can be already present in the wastewater to be treated or can be chemically added. The next step is the reduction of the nitrate and nitrite to nitrogen gas by means of denitrifying bacteria which use biodegradable organic matter as electron donor. However, there are some kinds of wastewater with low concentrations of biodegradable organic matter. In these cases an external biodegradable carbon source is necessary in order to obtain a complete denitrification, which implies an economic cost. Some typical external carbon sources are short chain alcohols (e.g. methanol, ethanol), acetate and glucose. Usually, methanol is the

cheapest available carbon source, thus it is the most used compound. The need of organic matter per unit of mass of nitrogen is about 3.7 g COD (g $N)^{-1}$ when methanol is employed.

The use of technologies based on anaerobic ammonium oxidation (Anammox) is a relatively new alternative for nitrogen removal from wastewaters with low COD/N ratios. This process is carried out by autotrophic bacteria which, in anoxic conditions, combine ammonium and nitrite into nitrogen gas and a small amount of nitrate. Since nitrite is not usually present in wastewater, it will be necessary to oxidize about 50% of the ammonium into nitrite (partial nitrification).

Partial nitrification and Anammox processes can be carried out in two different units. The first reactor is operated under aerobic conditions in order to convert approximately half of the ammonium in the influent into nitrite. The second reactor is the Anammox anoxic reactor where autotrophic denitrification is obtained.

Partial nitrification and Anammox processes can also be carried out together in a single unit. This technology has received different names: CANON (Completely Autotrophic Nitrogen removal Over Nitrite); OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) and deammonification. The main difference among them is that CANON process employs suspended biomass growing in a mixed reaction medium, while OLAND and deammonification are biofilm processes, thus biomass is growing on biodiscs or on moving plastic carriers, like Kaldnes rings. Dissolved oxygen concentration is the main operational variable to obtain a stable operation of the system. Diffusional limitations allow oxygen to be completely consumed in the outer layer of the granule or biofilm, the inner part being anoxic. Therefore, partial nitrification is carried out in the external part while Anammox bacteria will be growing in the inner layers.

In any case and regardless of the reactor configuration, both processes, nitrification and Anammox, are autotrophic, thus biodegradable organic matter is not necessary. Besides, the requirements of oxygen are about 40% lower compared to conventional nitrification/denitrification because only 50% of the ammonium needs to be oxidized to nitrite. The production of sludge is also much lower due to the small biomass yield of the autotrophic organisms. Therefore partial nitrification/Anammox can be considered cheaper and more sustainable than conventional nitrification/denitrification.

Despite the advantages of the Anammox process, there are still some important research issues which should be addressed in order to increase the chances to apply the Anammox process at full scale. Some of these issues are the focus of the research developed in the present doctoral thesis. Specifically, these topics are the improvement of the biomass retention in the reaction systems, the evaluation of the effects of the potential inhibitors of the process and the application of the process at the mesophilic range of temperature.

Along the next paragraphs, the main contents of each chapter of the thesis are summarized:

In **Chapter 1**, a literature revision about nitrogen removal from wastewater is done. Besides, the main characteristics and operational conditions of the Anammox process are described in detail. Economic analyses of different technologies for nitrogen removal from wastewater are also included. Finally, the future perspectives of nitrogen removal by Anammox are discussed.

In **Chapter 2**, the materials and methods employed to carry out the experimental work of this doctoral thesis are explained. First, the methods employed to analyze the properties of the liquid phase are detailed. They are followed by the techniques for the physical and

microbiological characterization of the biomass. Finally, the composition of the Anammox mineral medium is included. This medium was used to prepare the synthetic Anammox feeding.

In **Chapter 3**, the inhibition of the Anammox process caused by its substrates (nitrite and ammonium) is studied. The unionized species of the substrates (i.e. Free Ammonia (FA) and Free Nitrous Acid (FNA)) were assumed to be the responsible for the inhibition of Anammox process. An advantage of using inhibition threshold concentration levels in terms of these unionized compounds is that their use in the control of different Anammox systems with varied conditions may be easier.

In a first part, short time inhibitory effects were assessed by means of specific activity tests. Two different types of Anammox biomass (biofilm growing on inorganic carriers and flocculent sludge) were employed. The value of IC50 (concentration which caused 50% of inhibition) for FA was estimated to be about 35 mg NH_3-N L^{-1}, without significant differences between the two kinds of biomass tested. In the case of FNA, the value of IC50 for biofilm biomass was about 11 µg HNO_2-N L^{-1}. However, the flocculent biomass was much less resistant and its specific activity sharply decreased below 30% in the presence of only 4.4 µg HNO_2-N L^{-1}. Subsequently, the study of the long-term effects was carried out in lab-scale Sequencing Batch Reactors inoculated with the biofilm biomass. It was found that the long term effects were more important than those observed at short term exposition. In particular, at concentrations of 35-40 mg NH_3-N L^{-1} and 1.5 µg HNO_2-N L^{-1} stable operation of the reactor was not reached.

The aim of **Chapter 4** is to assess if the Anammox treatment of wastewaters from anaerobic digestion of manure was possible. These wastewaters are expected to contain significant concentrations of antibiotics. Thus, this chapter is focused on the effects caused by two broad spectrum antibiotics (chloramphenicol and tetracycline

hydrochloride) on the Anammox process. First, the short term effects were studied by means of activity and biotoxicity assays. It was found that both antibiotics produced strong inhibitory effects and besides, tetracycline hydrochloride caused deactivation of the biomass. Anammox activity and biotoxicity assays gave similar values of IC50 for chloramphenicol, about 400 mg L^{-1}. However, in the case of tetracycline hydrochloride, the IC50 calculated from activity tests was about 220 mg L^{-1}, much higher than 94 and 42 mg L^{-1}, which were the IC50 values at 5 and 15 min obtained by biotoxicity tests. The difference between biotoxicity results at 5 and 15 min would confirm the increase of the toxic effect caused by tetracycline along time.

Subsequently, long term effects were researched adding each antibiotic to the feeding of an Anammox SBR reactor. It was observed that 20 mg L^{-1} of chloramphenicol caused 80% of decrease of the Anammox activity. Similar effects were observed when the reactor was operated in presence of 50 mg L^{-1} of tetracycline hydrochloride. Despite the loss of activity, both antibiotics did not cause changes in the physical properties of the biomass, which allowed good biomass retention. Since these antibiotics were negatively affecting the Anammox process, degradation technologies which could be used previously to the Anammox reactor are discussed in this chapter.

The Anammox process has been usually operated, both at lab scale and industrial scale, to treat wastewaters at temperatures around 30 ºC. These wastewaters are usually the effluent of anaerobic digesters operated at the mesophillic range of temperature. In **Chapter 5** the operation of the process at lower temperatures is studied in order to apply the process to different types of wastewaters. In the first part, the short term effects of temperature were studied by means of specific activity tests. An Arrhenius type trend was found with activation energy of 63 kJ mol^{-1} and the optimum activity at 35-40 ºC. The next part of the experiments consisted of the gradual diminution of the temperature of an Anammox SBR, in

order to find a possible adaptation of the biomass. The system was efficiently operated at 18 ºC and the resistance of the biomass, which was slowly adapted to low temperatures, was higher than that observed during activity tests. However, when temperature of operation was 15 ºC, nitrite (i.e. limiting substrate) started to accumulate in the reaction medium and the system lost its stability. Finally, the supernatant of an anaerobic digester was treated at 20 ºC by means of a system with two SBR reactors in series, which were carrying out the partial nitrification and the Anammox process. The global nitrogen removal rate of this system was 0.08 g N $(L\ d)^{-1}$.

After researching independently the effects caused by the substrates and the temperature on the Anammox process, in **Chapter 6** a statistical tool is employed (response surface models) in order to assess the combined influence caused by several variables. In this case the biomass samples were taken from a deammonification reactor (one-stage biofilm Anammox process) and specific Anammox activity was employed as response variable. Temperature, pH, ammonium concentration, total nitrogen concentration and free ammonia to free nitrous acid ratio were chosen as the controlled variables. It was observed that the significant parameters in order to optimize the process were the temperature, the value of pH and the ratio between free ammonia and free nitrous acid. Besides, the optimum ranges of these variables were found. Taking into account this information, a control strategy for a deammonification reactor was developed.

Due to the slow growth rate and small biomass productivity of the Anammox process, it is important to mantain good biomass retention in Anammox reactors. This is especially important during start up when the amount of available inoculum is small. One of the alternatives studied to improve the retention of Anammox biomass was the use of biofilm reactors. The two last chapters of this thesis are focused on this technology. In **Chapter 7** the influence of mechanical stress and salinity

on formation of Anammox biofilms is studied. The development of the biofilms was monitored by means of a sensor based on surface vibration properties. It was observed that Anammox biomass has a good ability to form biofilms and the initial phase of adhesion to the support lasted about 5-7 days at the three different flow rates tested (25.2; 8.4; 7.3 L h^{-1}), corresponding to Reynolds numbers 188, 63 and 54. The stability of the biofilm was higher when it was formed under high mechanical stress. Besides, it was observed that the presence of salts (NaCl, $CaCl_2$) enhanced the formation of the biofilm because of the reduction of electrostatic repulsion forces. The effects of the $CaCl_2$ were stronger than those caused by NaCl probably because divalent cationic bridging was taking part when the calcium salt was used. Incorporation of inorganic compounds into the biofilm was observed in both cases.

Finally, in **Chapter 8**, the start up and operation of an Anammox biofilm system is studied. Natural zeolite was employed as the biofilm support because this material is able to adsorb ammonium, which is one of the substrates consumed by the Anammox organisms. This would favour the access of attached biomass to NH_4^+ and, therefore, it would promote the biofilm formation. It was observed that it was very important to keep a low concentration of ammonium in the reaction medium in order to promote the formation and growth of the biofilm. In these conditions, the most part of the ammonium was adsorbed on the zeolites.

Once the biofilm was established and developed, the retention of biomass in the system was very good, with volatile suspended solids concentrations in the effluent lower than 3 mg VSS L^{-1}. As a consequence, the biomass concentration in the reactor increased significantly. Besides, the specific activity of the biomass increased, reaching values about 0.5 g N (g VSS d)$^{-1}$.

The high density of zeolite particles implied the necessity of applying a high mixing power into the reactor to keep them in suspension. During

the start-up period, the applied stirring power would promote abrasion produced by bare zeolite particles which can cause detrimental effects on biofilm. Furthermore, the shear stress by itself could cause a decrease on the biomass specific activity. Once the particles were fully covered with biomass this problem was minimized due to the decrease of global particle density and, therefore, lower mixing power was needed. Furthermore, particles covered by biofilm had lower abrasive capacity.

Since the growth of biofilm was achieved when the ammonium concentration in the bulk liquid was relatively low, a reactor with this technology should be fed with a substrate ratio near to the stoichiometric one. In order to achieve this ratio, the previous partial nitrification reactor producing the nitrite should be carefully controlled. This might be difficult during the start up periods. One additional inconvenient might be the fact that, since Anammox can be inhibited by nitrite (according to Chapter 3), the operation with a significant excess of ammonium would be considered safer, especially if the influent concentrations are not very stable. However, since an amount of ammonium would be adsorbed on the solid support along the operation, the system may act like an "ammonium buffer" and nitrite overloads may be mitigated in some extent. Actually, this can be one of the main advantages of the use of zeolites when compared to other Anammox biofilm technologies (like biofilm on Kaldnes rings), since zeolites are a kind of "active support" while plastic, glass or other kinds of biofilm supports do not have that ability.

With the different works carried out and reported along the present doctoral thesis, the knowledge about some of the key aspects of the Anammox process has been increased. Therefore it is considered that the obtained knowledge, which has been summarised along the present section, will make easier the industrial implementation of the process in order to treat different kinds of wastewaters.

Chapter 1

Introduction

SUMMARY. In this first chapter, the motivation of the thesis is detailed. This work has been focused on the removal of nitrogen water pollution. It is well known that the nitrogen cycle is affected by anthropogenic activities and the release of ammonium in natural systems causes eutrophication (i.e., proliferation of algae and oxygen depletion in natural waters). This is the main reason why nitrogen has to be removed in Wastewater Treatment Plants (WWTPs). The traditional way to perform this removal is a conventional nitrification-denitrification process with different reactor configurations and technologies.

More stringent effluent quality requirements and the need for sustainable treatments seriously challenged the efficiency of the classical processes. Recently Anammox has arisen as a new process able to improve the nitrogen removal from wastewater. Some of the basic research about Anammox (stoichiometry, thermodynamics, biochemistry...) has been done in the last years. Furthermore, some full scale plants have been started up. However, Anammox still has some limitations and drawbacks which need to be addressed. With the worldwide full scale applications of the new technologies based on the Anammox process, not only environmental benefits are expected, but also economic. And the research presented in this thesis aims to increase the possibilities of Anammox full scale implementation.

1.1. WATER SHORTAGE AND POLLUTION: A GLOBAL PROBLEM

Water is essential for all known forms of life. It covers about 71% of the Earth surface and 97% of this surface water corresponds to the oceans (CIA, 2009). However, only groundwater and fresh water are useful for human consumption and both sources combined represent less than 1% of the total water on the planet. Although water is a renewable resource through water cycle (Figure 1.1), the available amount is becoming scarce.

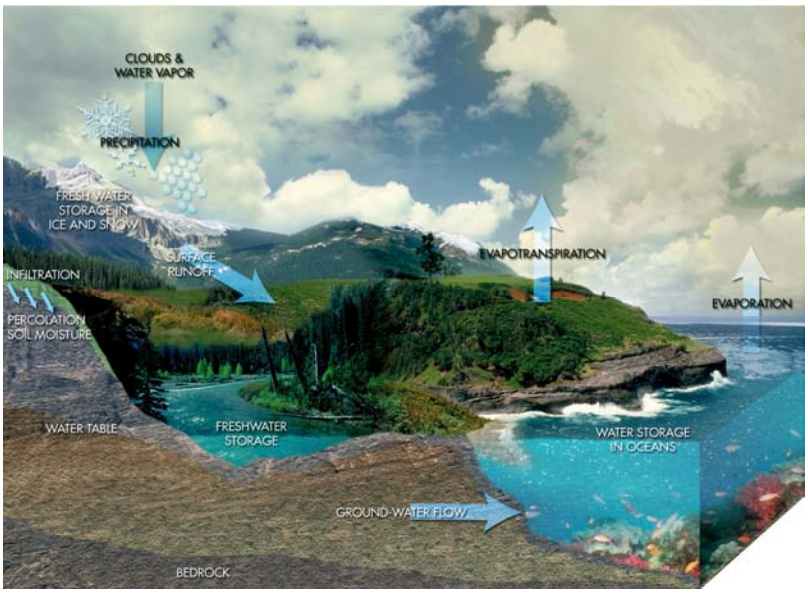

Figure 1.1. Water cycle in nature (picture by NASA Goddard Space Flight Center).

The world's supply of clean, fresh water is gradually decreasing. Water demand exceeds supply in many parts of the world and as the world population continues to increase, so does the water demand. In the developing world, almost all wastewater still goes untreated into local rivers and streams (UNEP International Environment, 2002).

Farmers use water for irrigation and some important part of this irrigation is considered as unsustainable (WBCSD, 2009). Besides, industry and

domestic or municipal uses of water are also very important and they are continuously growing because of the increase in the world population. Therefore, 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, specifically organic matter and nutrients like nitrogen and phosphorous, is performed in Wastewater Treatment Plants (WWTPs) and it is the object of intensive research.

1.2. LEGISLATION

Due to the raising general concern about water pollution, new laws were developed to control the quality of the effluents. Besides, eutrophication effects became a serious global problem, thus there is a need to prevent or reduce the negative impacts of nutrients on the environment.

Considering legislation framework affecting Spain, European Union law is one of the main normative sources.

The Council Directive on Urban Waste Water Treatment (Council Directive 91/271/EEC) concerns the collection, treatment and discharge of urban wastewater and the treatment and discharge of wastewater from certain industrial sectors. Its aim is to protect the environment from any adverse effects due to discharge of such waters. To achieve this, member states shall ensure that wastewater entering the collecting systems is treated by a biological process (secondary) or equivalent, before discharge. Moreover, application of more stringent treatment technologies (tertiary) is required in defined sensitive areas. The Directive also allows the establishment of less sensitive coastal areas, for which primary treatment would be sufficient, if it can be shown that there is no adverse impact on the environment (Art. 6; Council Directive 91/271/EEC). Member states had to establish lists of sensitive areas. It has been estimated that in 2004

about 34% of the pollutant load from wastewater that falls under the scope of the directive is discharged into sensitive areas (European Commission, 2004).

This Directive was amended by the Commission Directive 98/15/EC in order to clarify the requirements in relation to discharges from urban WWTPs 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 organic matter, nitrogen and phosphorus in purified wastewater discharged to the rivers and other water bodies.

Table 1.1. Requirements for discharges from urban WWTPs to identified sensitive areas subject to eutrophication.

Parameter	Concentration[1]	Minimum removal (%)
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 p. e.) 10 (> 100,000 p. e.)	70-80
Total Phosphorus (mg P L^{-1})	2 (10,000-100,000 p. e.) 1 (> 100,000 p. e.)	80

[1]Council Directive 91/271/EEC amended by the Commission Directive 98/15/EC. BOD_5 stands for Biodegradable Oxygen Demand at 5 days; COD stands for Chemical Oxygen Demand.

1.3. THE NITROGEN CYCLE AND NITROGEN POLLUTION

Nitrogen is an element necessary for the live organisms, accounting for approximately 6% of their dry mass (Bonete *et al.*, 2008). Therefore, the performance of every step of the nitrogen cycle has an important effect on these organisms. About 80% of the atmosphere is nitrogen, being its largest pool. Fixation of inorganic nitrogen is a key process of the nitrogen cycle which can be carried out by higher plants (Hageman and Reed, 1980), algae (Solomonson and Vennesland, 1972), yeast (Sengupta *et al.*, 1996) and bacteria (Moreno-Vivián *et al.*, 1999). Inorganic nitrogen can be found in nature in redox states from +5 (nitrate) to -3 (ammonia), but in organic compounds produced by living organisms it is almost exclusively present in redox state -3, being part of two of the most important biological molecules: nucleic acids and proteins (Richardson and Watmough, 1999). The reactions of the biogeochemical nitrogen cycle allow the conversions among the different nitrogen compounds by oxidative and reductive processes (Figure 1.2).

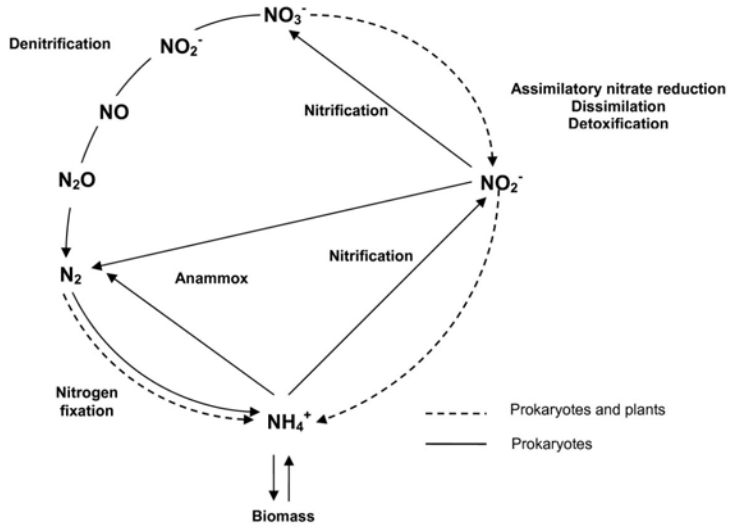

Figure 1.2. Nitrogen cycle scheme (Bonete *et al.*, 2008).

Some years ago it was assumed that the microbial nitrogen cycle was complete and no new involved organisms existed in nature (Strous and Jetten, 2004). However, relatively recent discoveries showed that the complete understanding of this cycle was far from complete. Among these discoveries, probably the more outstanding were the anaerobic ammonium oxidation (Anammox) (Strous *et al.*, 1999) and the ammonium oxidation by some Archaea (Konneke *et al.*, 2005). With the discovery of Anammox bacteria, new efficient alternatives to remove nitrogen in WWTP appeared. Besides, the knowledge about nitrogen gas production in the oceans changed substantially. After Anammox bacteria revealed to be prevalent in anoxic deep marine environments (Kuypers *et al.*, 2003; Kuypers *et al.,* 2005) it was postulated that Anammox could be the main process responsible for nitrogen gas production in the oceans. However Revsbech *et al.* (2006) reported that the N_2 production by the Anammox process strongly varies depending on the location. Therefore, current estimations report that Anammox may be responsible for 30–50% of global production of N_2 in the oceans.

Humans are altering the global cycle of nitrogen (Figure 1.2) basically due to: i) nitrogen fixation as a result of intensive agriculture (especially in the cultivation of soy, alfalfa and clover), ii) chemical fixation by the Haber-Bosch process in the production of chemical fertilizers and iii) pollution emitted by vehicles and industrial plants. The two first activities (i.e. agriculture and chemical fixation) are the responsible for doubling the annual transfer of nitrogen into biologically available forms (Vitousek *et al.*, 1997).

The alteration of the nitrogen cycle caused by the anthropogenic activities has certainly satisfied essential requirements to sustain an increasing global population, such as providing enough food. However, the high levels of agricultural intensification reached are damaging the environment (Galloway *et al.*, 2008).

Only a small part of the total amount of inorganic nitrogen released to the environment is transformed to N_2 by natural means. As a result, the total income of nitrogen to the biosphere is much larger than the nitrogen removal rate via denitrification. Thus an increasing eutrophication can be expected in water bodies worldwide. Furthermore, the presence of anthropogenically generated nitrogen compounds in the environment can create important problems of toxicity.

1.3.1. Toxicity of nitrogen compounds and eutrophication

Ammonium, nitrite and nitrate released to the water bodies can be toxic and produce eutrophication.

Ammonia can have toxic effects on aquatic life, for example fish mortality by short exposure to high concentrations and sterilization or mutagenesis when exposure is long. Concentrations of free ammonia as low as 0.03 mg NH_3-N L^{-1} were found to be toxic for aquatic organisms (Solbé and Shurben, 1989).

The main toxic effects caused by nitrite and/or nitrate are:

- Methemoglobinemia, a disease caused by the presence of unusually high concentrations of methemoglobin in blood. Methemoglobin is a form of hemoglobin that does not bind oxygen; thus, when its concentration is high, tissue hypoxia takes place. Ingestion of nitrite and nitrate is medically linked to methemoglobinemia, which typical symptom is the bluish appearance of the skin (when happening to babies it is called blue baby syndrome).
- Formation of carcinogenic organic molecules with the amine and amide groups.
- Formation of NO_x, gases contributing to the destruction of the ozone atmospheric layer.

Eutrophication is an increase in the concentration of chemical nutrients (mainly, nitrogen and phosphorus) in an ecosystem to an extent that increases the rate of accumulation of energy with the fixation of sunlight by plants, algae and other autotrophic organisms. Depending on the degree of eutrophication, subsequent negative environmental effects such as anoxia and severe reductions in water quality, fish, and other animal populations may occur.

The substrates required for plants and 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.

Eutrophication is frequently a result of nutrient pollution, such as the release of sewage effluent, storm water run-off and water carrying excess fertilizers into rivers or lakes. Eutrophication generally promotes excessive plant growth and decay, favours certain weedy species over others, and may cause a severe reduction in water quality. In aquatic environments, fast growth of invasive aquatic vegetation or phytoplankton (algal blooms) disrupts the normal functioning of the ecosystem, causing problems such as a lack of oxygen in water, needed for fish and shellfish to survive. The water then becomes cloudy and coloured. Human society is impacted as well: eutrophication decreases the resource value of rivers, lakes, and estuaries so the recreation, fishing, hunting and aesthetic enjoyment are hindered. Health-related problems can occur where eutrophic conditions interfere with drinking water treatment (Bartram *et al.*, 1999), especially when potentially toxic cyanobacteria appear. Cyanotoxins produced by these bacteria may cause human poisoning or skin allergies, when swimming in waters with cyanobacterial bloom.

1.4. NITROGEN REMOVAL FROM WASTEWATER: TECHNOLOGIES BASED ON NITRIFICATION AND DENITRIFICATION

1.4.1. Nitrification/Denitrification

The conventional processes to remove nitrogen from wastewater involve nitrification and denitrification steps.

Nitrification is the autotrophic oxidation of ammonium into nitrate. It is an aerobic process with two sequential phases: the oxidation of the ammonium to nitrite and the oxidation of this nitrite to nitrate. Each step is carried out by different bacterial groups: Ammonium Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB). Some of the best known AOB are genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*. Some NOB genera are *Nitrospira*, *Nitrospina*, *Nitrococcus*, *Nitrocystis* and the best known, *Nitrobacter*.

Catabolism of ammonium oxidation can be described by Equation 1.1:

$$NH_4^+ + 1.5\ O_2 + 2\ HCO_3^- \rightarrow NO_2^- + 3\ H_2O + 2\ CO_2 \quad (1.1)$$

If the cellular growth is taken into account, the stoichiometry would be:

$$NH_4^+ + 1.382\ O_2 + 1.982\ HCO_3^- \rightarrow 0.018\ C_5H_7O_2N + 0.982\ NO_2^- + 2.927\ H_2O + 1.891\ CO_2 \quad (1.2)$$

And for the oxidation of nitrite:

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (1.3)$$

If bacterial growth is included in Equation 1.3:

$$NO_2^- + 0.003\ NH_4^+ + 0.488\ O_2 + 0.010\ H_2CO_3 + 0.003\ HCO_3^- \rightarrow 0.003\ C_5H_7O_2N + NO_3^- + 0.008\ H_2O \quad (1.4)$$

Yields for the most common AOB and NOB, *Nitrosomonas* and *Nitrobacter*, are 0.15 mg_{cells} (mg NH_4^+-N)$^{-1}$ and 0.02 mg_{cells} (mg NO_2^--N)$^{-1}$, respectively.

Total consumption of oxygen in order to convert ammonium into nitrate is about 4.2-4.5 g O_2 (g NH_4^+-N)$^{-1}$. Furthermore, nitrification causes alkalinity consumption and 7.1 g $CaCO_3$ (g NH_4^+-N)$^{-1}$ are necessary. This alkalinity can be already present in the wastewater to be treated or can be chemically added.

The second step of the nitrogen removal process is denitrification, which is carried out by heterotrophic organisms in anoxic conditions. During this process, nitrate and/or nitrite formed during nitrification are reduced to N_2 by organisms which employ organic matter as a source of carbon and energy. Denitrifying organisms commonly belong to *Alphaproteobacteria* or *Betaproteobacteria*, like *Pseudomonas*, *Alcaligenes*, *Paracoccus* and *Thiobacillus*. Besides, some halophilic *Archaea* are also able to perform denitrification. Equation 1.5 shows the global stochiometry of denitrification process when acetic acid is the carbon source (Vázquez-Padín, 2009).

$$5\ CH_3COOH + 8\ NO_3^- \rightarrow 10\ CO_2 + 4\ N_2 + 6\ H_2O + 8\ OH^- \quad (1.5)$$

And when the equilibrium of carbon dioxide is included:

$$0.625\ CH_3COOH + NO_3^- \rightarrow HCO_3^- + 0.25\ CO_2 + 0.5\ N_2 + 0.75\ H_2O \quad (1.6)$$

According to the stoichiometry it can be observed that denitrification produces alkalinity (Equation 1.6), thus it is able to compensate, in some extent, the consumption of alkalinity caused by the nitrification.

When biodegradable organic matter is not present in the wastewater to be treated or when its concentration is not enough in order to complete denitrification, the addition of an external carbon source is necessary. Some typical electron donors are small chain alcohols (methanol, ethanol), acetate and glucose. Usually, methanol is the cheapest available carbon source, thus it is the most used compound (Park and Yoo, 2009). The need of organic matter per unit of mass of nitrogen is about 3.7 g COD (g $N)^{-1}$ when methanol is employed.

1.4.2. Technologies

1.4.2.1. Activated sludge and biofilm systems

Activated sludge is the most popular technology for the biological treatment of domestic and industrial wastewaters, removing both organic matter and ammonium nitrogen. The predenitrification configuration is the most common, especially in small plants. Wastewater is initially led to a stirred denitrifying basin with no air supply to avoid the oxidation of organic matter, which may cause biodegradable COD limitation in the denitrifying zone. Afterwards wastewater is treated in a basin with aeration where nitrification occurs. 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 and on the recirculation ratio applied between both basins.

One of the main disadvantages of this process is that the organic matter coming from the denitrification unit causes the proliferation of heterotrophic bacteria in the aerobic unit. These microorganisms compete with nitrifying bacteria for oxygen, which concentration must be maintained at levels higher than 2 mg O_2 L^{-1} to avoid the failure of the nitrification process.

Some other technologies based on nitrification and denitrification are called Biodenitro, Biodenipho, UCT process and Bardenpho. They have

been developed basing on modifications of the initial two-chambers configuration and comprise higher number of chambers and/or recirculation streams with the objective of improving nitrogen and eventually phosphorus removal (Metcalf and Eddy, 2003).

Submerged granular biofilters constitute one of the most widely employed biofilm nitrification/denitrification technology. This technology is based on the use of reactors where wastewater flows through a fixed granular bed. This fixed bed usually has 3 to 4 meters height and it is aerated. Wastewater is forced to go up through the bed instead of flowing by gravity.

Granular carriers of the bed have a diameter between 3 and 8 millimetres and they are usually made of plastic or clay. They have a high specific area, where biofilm growths, allowing a high concentration of biomass per unit of volume of the reactor. There is a network of air diffusers on the bottom of the reactor in order to fulfil the requirements of oxygen. However, some parts of the reactor can be maintained anoxic, therefore nitrification and denitrification can be carried out in the same system.

There are two possible configurations of submerged biofilters. In the first, the removal of organic matter is previously performed in an aerobic biofilter. Then, nitrification and postdenitrification can be performed in a second biofilter. Methanol will be used as carbon source achieving very low nitrate concentrations in the effluent. This configuration allows a very easily controlled operation, but the operational costs are relatively high. One example is the WWTP of Oslo (Norway) (Sagberg *et al.*, 2006). In order to minimize costs it is also possible to use a predenitrification configuration. In this case denitrification is performed employing the organic matter present in the wastewater. Then the nitrification can be carried out in another separated biofilter or in the same, combining predenitrification in the lower part and nitrification in the upper part (Rother and Cornel, 2007).

1.4.2.2. Bioaugmentation

The concept of bioaugmentation has been applied in the University of Delft in order to develop the BABE (Bio Augmentation Batch Enhance) technology (Salem *et al.*, 2002; Salem *et al.*, 2004; Berends *et al.*, 2005). This system employs the effluent from the anaerobic sludge digester, with high ammonium concentration, to promote the growth of nitrifying biomass. Then the stream with the produced biomass enters the activated sludge unit (Figure 1.3), allowing a capacity increase of the nitrification unit. This can be useful in order to:

- Promote the nitrification in overloaded plants.
- Increase the denitrification in plants with total nitrification, because a bigger volume of the reactor can be used as anoxic.

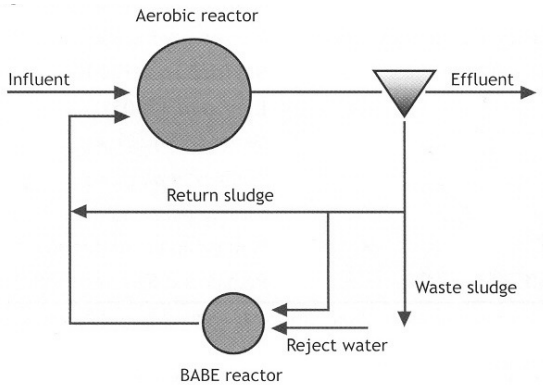

Figure 1.3. BABE process.

1.4.2.3. Partial nitrification: nitrogen removal via the nitrite route

Consumption of resources (oxygen and external carbon source, when needed) can be minimized by oxidizing the ammonium into nitrite instead of nitrate. Then, produced nitrite can be transformed into nitrogen gas. This strategy allows saving 25% of oxygen and 40% of organic carbon source (when external source of carbon is used) compared to nitrification/denitrification processes. Therefore, the specific oxygen consumption will be about 3.4 g O_2 (g NH_4^+-N)$^{-1}$ to perform the conversion

to nitrite and 2.3 g COD (g NH_4^+-N)$^{-1}$ will be necessary to remove this nitrite. An additional advantage of this technology is that the sludge production is 30% of that corresponding to nitrification/denitrification processes.

The partial nitrification/denitrification strategy has been implemented in different configurations of reactors. One of the most common is the Sharon (Single reactor system for High-activity Ammonia Removal over Nitrite) process developed by Hellinga *et al.* (1998). This process is carried out in a continuous stirred tank reactor with suspended biomass. The reactor is operated at temperatures between 30 and 40 °C and low sludge retention time (in this type of continuous reactor, the hydraulic retention time is equal to the sludge retention time). In these conditions, AOB are selectively retained while the slower growing NOB are washed out. Both nitrification and denitrification may take place in the same stirred reactor using intermittent aeration. This principle for the selective enrichment of the ammonium oxidizing bacteria is not only restricted to Continuous Stirred Tank Reactors (CSTR); partial nitrification-denitrification was also successfully implemented in Sequencing Batch Reactors (SBR) with Sludge Retention Time (SRT) control (Fux *et al.*, 2006; Dosta *et al.*, 2007).

1.5. THE ANAMMOX PROCESS

1.5.1. Discovery and stoichiometry

For a long time, it was thought that ammonium oxidation could only take place aerobically. In 1977 Broda predicted, using thermodynamic calculations, the existence of chemolitoautotrophic bacteria capable of oxidizing ammonium using nitrite as electron acceptor (Equation 1.7):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H2O \qquad -\Delta G° = 335\ kJ/(mol\ NH_4^+) \qquad (1.7)$$

That prediction was experimentally confirmed two decades later by Mulder (1995) in a denitrifying pilot plant, treating wastewaters from a

yeast plant. An ammonium loading rate of 0.4 g NH_4^+-N $(L\ d)^{-1}$ was removed in this system (Mulder *et al.*, 1995). These authors called the process ANAMMOX (ANaerobic AMMonium OXidation). The stoichiometry of the process was established by Strous *et al.* (1998) as follows:

$$NH_4^+ + 1.3\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow$$
$$1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \qquad (1.8)$$

Anammox uses nitrite as the electron acceptor in order to oxidize ammonium. Since nitrogen will be present in wastewaters in form of ammonium and according to the stoichiometry, the oxidation of about half of this ammonium into nitrite will be necessary to apply the Anammox process. The same processes for nitrification to nitrite described in Section 1.4.2.3 may be applied in this case, but controlling the extent of the ammonium conversion. Further discussion about partial nitrification can be found in Section 1.6.3.

1.5.2. Thermodynamics and kinetics of Anammox

The Anammox process is chemolitotrophic, which generally implies microorganisms characterized by low growth rates and yields due to the low Gibbs free energy involved in the reaction. In this case, the Gibbs free energy and the activation energy calculated by Strous (2000) are 357 KJ $(mol\ NH_4^+)^{-1}$ and 70 kJ $(mol\ NH_4^+)^{-1}$, respectively.

The kinetic parameters were calculated by Strous *et al.* (1998) for an enriched Anammox culture of the specie *Candidatus* Brocadia anammoxidans (Table 1.2). The low growing rates of these microorganisms imply, on one hand, long start-up periods for this kind of systems, and on the other hand, low amounts of sludge produced, solving the problem of the disposal of sludge in excess. These facts will imply the need of systems with a good biomass retention but also money savings.

Table 1.2. Kinetic parameters of *Candidatus* Brocadia anammoxidans (Strous *et al.*, 1998).

Parameter	Value
Substrate consumption rate	45 nmol NH_4^+ ($mg_{protein}$ min)$^{-1}$
Maximum growing rate	0.003 h^{-1}
Duplication time	10.6 d
Substrate affinity constant (nitrite)	< 5 µM
Substrate affinity constant (ammonium)	5 µM
Yield	0.066 mol C (mol NH_4^+)$^{-1}$
Free energy	-357 kJ (mol NH_4^+)$^{-1}$

1.5.3. Biochemistry

Van de Graaf *et al.* (1997) realized studies with markers to research the reaction mechanisms of the Anammox organisms. These authors employed different combinations of nitrogen compounds to test the formation and consumption of possible intermediate products. They found that these intermediates were hydrazine and hydroxylamine. Schalk *et al.* (1998) confirmed that hydrazine was an intermediate of the Anammox process. They hypothesized the mechanism which is shown in Figure 1.4. Ammonium and hydroxylamine would be combined into hydrazine, and then oxidized into N_2. This oxidation would generate 4 electrons used to reduce the nitrite into hydroxylamine. During the ammonium oxidation, low quantities of nitrate are produced from nitrite. This oxidation of nitrite into nitrate generates the electrons needed for the CO_2 fixation.

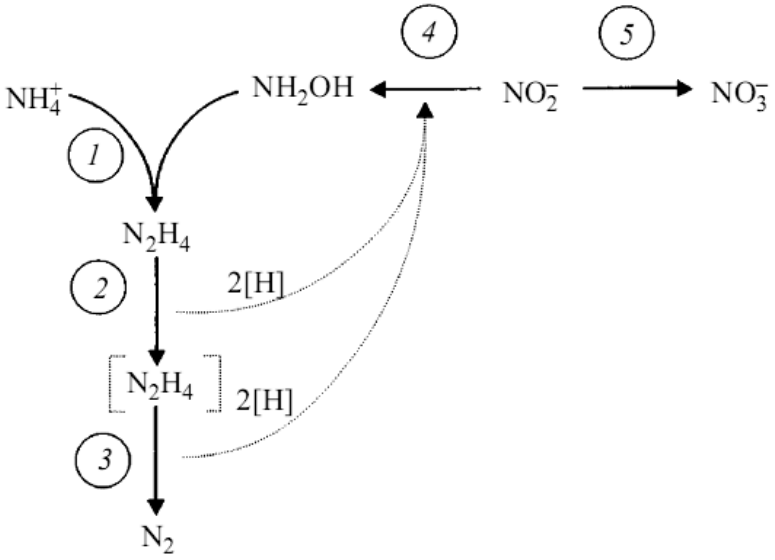

Figure 1.4. Proposed metabolic route of the Anammox process. (Schalk *et al.*, 1998)

Schalk *et al.* (1998) observed that if hydrazine was added to an Anammox culture, the obtained products were ammonium and N_2 in a 4:1 ratio, while with the addition of hydrazine and nitrite as electron acceptor the final product was N_2. These authors also observed that hydrazine was toxic in long-term periods and that it was not possible to grow Anammox directly from hydrazine and nitrite.

1.5.4. Microbiology

After the discovery of the Anammox process and the identification of the first Anammox organism, *Candidatus* Brocadia Anammoxidans, other species capable of carrying out the process were detected and their 16S rRNA sequences were determined: *Candidatus* Kuenenia stuttgartiensis, *Candidatus* Scalindua sorokinii, *Candidatus* Scalindua brodae, *Candidatus* Scalindua wagneri, *Candidatus* Brocadia fulgida and *Candidatus* Anammoxoglobus propionicus (Schmid *et al.*, 2000; Fujii *et al.*, 2002; Kuypers *et al.*, 2003; Schmid *et al.*, 2003; Kartal *et al.*, 2007; Kartal *et al.*, 2008).

The Anammox organisms resemble each other in the phylogenetic analyses of their 16S rRNA sequences (Figure 1.5), which show that they form a monophyletic branch, which consists of five distinct genera with about 90% sequence similarity to each other, within the phylum Planctomycetes. As the rest of the species within the order Planctomycetales, they lack of peptidoglycan, an almost universal polymer found within the Bacteria domain. Instead, protein is the major constituent of their cell walls. Among the Bacteria domain, this lack of peptidoglycan is a characteristic shared only with the Chlamydiae and the cell-wall-free Mycoplasms (Lindsay *et al.* 2001).

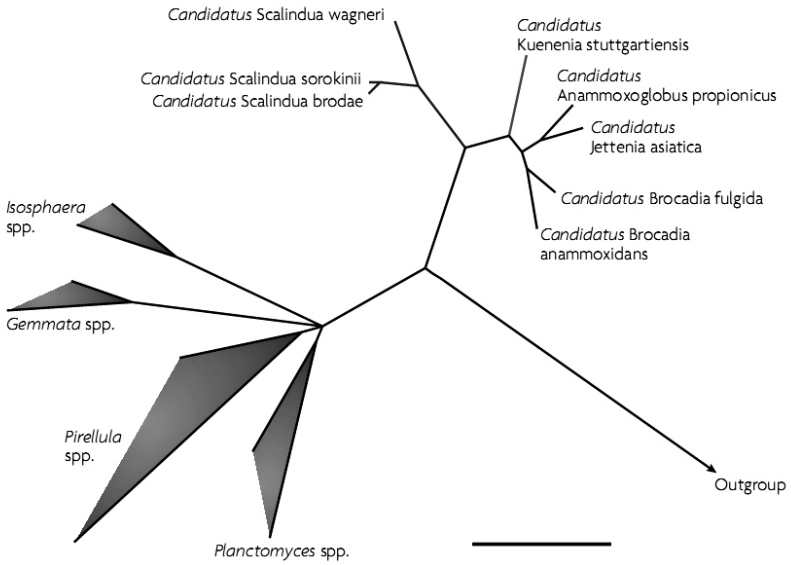

Figure 1.5. 16s ribosomal RNA phylogenetic tree of Anammox bacteria. It shows the relationships of the different families of Anammox bacteria. The scale bar represents 10% sequence divergence (adapted from Kuenen, 2008).

Another structural characteristic of Anammox bacteria is the presence of an organelle called anammoxosome (Figure 1.6) which occupies more than 30% of the cell volume (van Niftrik *et al.*, 2004). It has been found that the enzyme hydrazine oxidoreductase, which is responsible for the oxidation of the intermediate hydrazine (Section 1.5.3), is present

exclusively inside the anammoxosome. Furthermore, this compartment is surrounded by a membrane nearly entirely composed of unique ladderane lipids (Sinninghe Damste *et al*., 2002; van Niftrik *et al*., 2004).

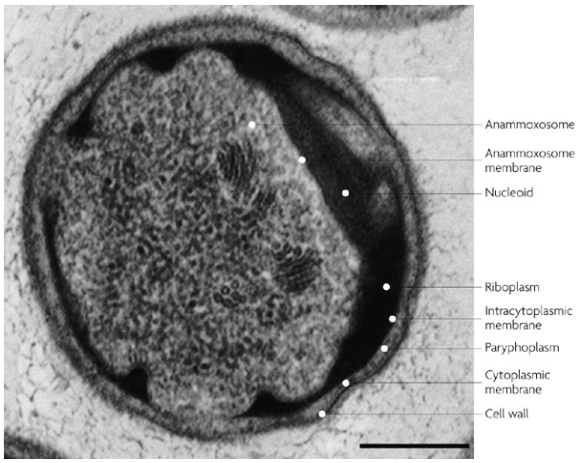

Figure 1.6. Microstructure of *Candidatus* Kuenenia stuttgartiensis with its cellular compartments, including the anammoxosome. The scale bar represents 200 nm. Adapted from Kuenen (2008).

1.5.5. Factors affecting the Anammox process

1.5.5.1. Effect of temperature

The specific substrate (ammonium and nitrite) consumption rates and nitrate production rate were estimated by Strous (2000) at different temperatures. This author observed that the optimum temperature for the Anammox biomass was 40 ± 3 ºC. Between 20 ºC and 37 ºC, the activity depends on the temperature according to the Arrhenius law. At a temperature of 10 ºC or lower the observed activity was null. Isaka *et al.* (2007) have reported that they could efficiently operate an Anammox reactor at 20-22 ºC with a relatively high biomass concentration of 20 g VSS L^{-1}. From their data, a stable Anammox activity about 0.4 g N (g VSS $d)^{-1}$ can be estimated.

Toh *et al.* (2002) tried to select and enrich an autotrophic anaerobic ammonium oxidation consortium from sludge collected in a municipal

treatment plant in Sydney, at two different temperatures, 37 ºC and 55 ºC. While mesophilic activities were successfully obtained in batch and continuous cultures, thermophilic Anammox organisms could not be cultivated at 55 ºC.

1.5.5.2. Effect of pH

Strous (2000) studied the Anammox activity dependence on the pH, finding a physiological interval of pH of 6.7–8.3. The optimum pH value for operation was around 8. Egli *et al.* (2001) observed Anammox activity even at a pH range of 8.5–9.0.

Ahn *et al.* (2004) studied the combination in the same reactor of anaerobic digestion and Anammox, for the treatment of piggery waste characterized by its high strength (56 g COD L^{-1} and 5 g TKN L^{-1}). They used a lab-scale up-flow anaerobic sludge bed reactor under mesophilic conditions and supplemented the raw piggery waste with nitrite. The pH of the effluent was about 9.3-9.5 (an important amount of alkalinity was produced due to the organic reduction in the anaerobic treatment of the piggery waste) but the Anammox activity in the reactor was not inhibited in spite of the pH values which were out of the ranges reported by Strous (2000) and Egli *et al.* (2001).

1.5.5.3. Effect of substrate and product concentrations

Strous (2000) exposed Anammox biomass to high ammonium and nitrate concentrations (up to 1 g N L^{-1}) in a SBR during one week and did not observe negative effects on the activity. Neverthless, this author found that the biomass (*Candidatus* Brocadia anammoxidans) completely lost its activity at concentrations of nitrite of 98 mg NO_2^--N L^{-1} (7 mM). Furthermore, if the biomass was subjected to nitrite concentrations higher than 5 mM (70 mg NO_2^--N L^{-1}) for a period of 12 h, the process was totally inhibited.

Egli *et al.* (2001) showed that *Candidatus* Kuenenia stuttgartiensis has a relatively important tolerance to nitrite concentrations up to 180 mg NO_2^--N L^{-1}. On the contrary, Fux *et al.* (2002) reported an inhibition of the Anammox process in a pilot plant fed with the effluent of a Sharon reactor (section 1.4.2.2) that treated a sludge digester effluent. In this case, the organisms (*Candidatus* Kuenenia stuttgartiensis) were strongly inhibited by a nitrite concentration of 60 mg NO_2^--N L^{-1}. The activity was slowly restored two weeks after the influent nitrogen load was reduced to nearly 50% of the initial value.

A more recent work studied the short-term inhibitory effects of both substrates and nitrate on the Anammox activity (Dapena-Mora *et al.*, 2007). These authors estimated the 50% inhibitory concentration (IC50) for each compound (Table 1.3).

Table 1.3. IC50 of ammonia, nitrite and nitrate (Dapena-Mora *et al.*, 2007).

Compound	IC50 (mg N L^{-1})
Ammonium	770
Nitrite	350
Nitrate	630

1.5.5.4. Effect of oxygen

Van de Graaf *et al.* (1996) and Strous *et al.* (1997a) demonstrated that oxygen reversibly inhibits the Anammox process. These authors did not observe Anammox activity in microaerobic conditions (2.0, 1.0 and 0.5% of air saturation). Nevertheless, Sliekers *et al.* (2002 and 2003) operated an Anammox SBR and a gaslift reactor under oxygen-limited conditions and, although the maximum Anammox activity decreased, a stable coexistence of Anammox and aerobic ammonium oxidizers permitted a completely autotrophic removal of nitrogen in both systems. In this case, the existence of Anammox in presence of oxygen is possible because it is

consumed by the nitrifying organisms. In those systems, nitrite oxidizing bacteria growth (and subsequent nitrate production) is prevented due to the lower affinity for oxygen compared to ammonia oxidizing bacteria and for the lower affinity for nitrite compared to Anammox bacteria.

1.5.5.5. Effect of exogenous compounds

Van de Graaf *et al.* (1995) studied the effect of antibiotics and other possible inhibitors on the Anammox activity in batch cultures. These authors showed that the addition of 800 mg L^{-1} of ampicillin inhibited ammonium removal almost completely, while a concentration of 400 mg L^{-1} of this compound reduced the activity by 71%. Other tested compounds, such as 2,4-dinitrophenol, $HgCl_2$ and carbonyl cyanide m-chlorophenylhydrazone (CCCP), resulted to be strong inhibitors of the anaerobic ammonium oxidation (inhibition of 95% of the activity at concentrations of 1 mM or lower).

Van de Graaf *et al.* (1996) started up a Fluidized Bed Reactor (FBR) fed with ammonium and nitrite, using sand as support material. With the enriched sludge they carried out a series of batch experiments, adding different chemicals to the medium. These authors found that both organic (acetate, propionate, glucose, fructose and lactose) and inorganic (sulfide, sulfur, sulfite, thiosulfate) electron donors had no effect or increased the ammonium oxidation rate when added at low concentrations (1-5 mM) but, in continuous assays, the organic compounds had a negative effect because they favour the growth of heterotrophic bacteria. Nevertheless, Güven *et al.* (2005) showed that Anammox bacteria could compete successfully with heterotrophic denitrifiers for propionate, because they can carry out propionate oxidation simultaneously with anaerobic ammonium oxidation.

A more recent work by Dapena-Mora *et al.* (2007) found that the presence of salts, phosphate or flocculant at relatively high concentrations (5.8 g NaCl L^{-1}; 7.5 g KCl L^{-1}; 2.7 g KH_2PO_4 L^{-1}; 500 mg flocculant L^{-1}) does not

affect the process. However, to guarantee the proper operation of the Anammox process the organic matter and sulphide must be absent.

1.5.5.6. Effect of shear stress

Arrojo *et al.* (2006) have reported that shear stress can cause a decrease in the activity of Anammox biomass. According to their work, despite Anammox sludge is highly resistant to mechanical stress, it would be not advisable to operate a SBR with specific mechanical stirring power higher than 0.090 kW $(m^3)^{-1}$. Moreover, Arrojo *et al.* (2008) found that Anammox biomass is less resistant to shear stress caused by gas-flow mixing. In this case these authors advise not to operate with specific input power higher than 0.017 kW $(m^3)^{-1}$.

1.6. ANAMMOX TECHNOLOGIES

1.6.1. Two stages configuration

Partial nitrification and Anammox processes can be carried out in two different units. The first reactor is operated under aerobic conditions in order to convert approximately half of the ammonium in the influent into nitrite. The second reactor is the Anammox anoxic reactor where autotrophic denitrification is obtained. The most common system to reach partial nitrification is the Sharon reactor described in Section 1.4.2.3 (Hellinga *et al.*, 1998) (Figure 1.7). This reactor is operated under aerobic conditions and at temperatures over 30 ºC, controlling the hydraulic retention time to wash out NOB but keeping AOB. Usually the employed HRT is 1 d (Mosquera-Corral *et al.*, 2005). By controlling dissolved oxygen and pH in the system, it is possible to keep the optimum ammonium/nitrite ratio in the effluent (Volcke *et al.*, 2006; van Hulle *et al.*, 2007).

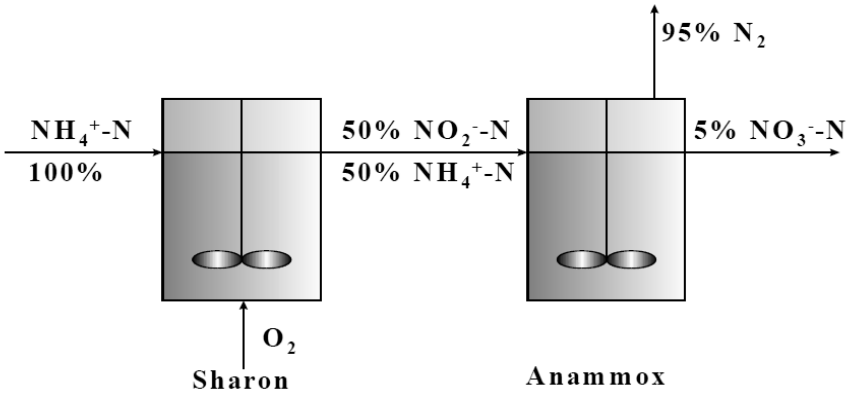

Figure 1.7. The Sharon-Anammox combined system.

Partial nitrification can also be carried out in a sequencing batch reactor (SBR) operated at relatively high temperature and controlled SRT. Gali *et al.* (2007) performed a comparative study to produce the correct influent for Anammox process from anaerobic sludge reject water. They demonstrated that both systems (SBR and Sharon chemostat) were able to achieve the same specific conversion rate (40 mg NH_4^+-N (g VSS h)$^{-1}$) but the SBR achieved a higher value of absolute nitrogen removal (1.1 g N (L d)$^{-1}$ versus 0.35 g N (L d)$^{-1}$), due to the different HRT used. The Sharon process showed however, a better stability. One more recent option is the use of aerobic granules in order to perform the conversion of half of the ammonium into nitrite (Vazquez-Padin, 2009). This work reported stable partial nitrification operating a granular SBR at room temperature. The average size of the developed granules was about 3 mm and the reactor was operated along one year. In this case the two key aspects to obtain partial nitrification were the oxygen diffusion limitation into the granule and the controlled dissolved oxygen concentration in the bulk liquid (2.0-3.5 mg O_2 L^{-1}).

When effluents from anaerobic digesters are treated, the optimum ammonium/nitrite ratio can be achieved without any special control system, because these effluents have an ammonium/bicarbonate molar ratio equal to 1. Since each mol of ammonium oxidized consumes 2

moles of bicarbonate, when 50% of the present ammonium is oxidized, bicarbonate will be fully depleted. This will cause a pH drop which stops nitrification process.

The treatment after partial nitrification, i.e. the Anammox step, can be carried out in different reactors like UASB (Upflow Anaerobic Sludge Blanket), similar to the ones used in the anaerobic digestion processes; gas-lift; continuous stirred tanks; etc. At laboratory scale, the SBR is widely used due to its flexibility of operation and easy control. Dapena-Mora *et al.* (2004) showed that the SBR is a suitable system to grow and enrich Anammox biomass in the form of granular sludge. Recently, SBRs are also being employed as full scale Anammox reactors, but in this case with one stage configuration (Section 1.6.2).

1.6.2. One stage configuration

Partial nitrification and Anammox processes can be carried out together in a single unit. This technology has received different names: CANON (Completely Autotrophic Nitrogen removal Over Nitrite; Third *et al.*, 2001); OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification; Kuai and Verstraete, 1998) and deammonification (Hippen *et al.*, 1997; Helmer *et al.*, 2001). The main difference among them is that CANON process employs suspended biomass growing in a mixed reaction medium, while OLAND and deammonification are biofilm processes, thus biomass is growing on biodiscs or on moving plastic carriers, like Kaldnes rings. Deammonification moving bed reactors are operating in Hattingen (Germany) and Himmerfjarden (Sweden) (Section 1.8.2.1).

Dissolved oxygen concentration is the main operational variable to obtain a stable performance of the system. Diffusional limitations allow oxygen to be completely consumed in the outer layer of the granule or biofilm, the inner part being anoxic. Therefore, partial nitrification is carried out in the external part while Anammox bacteria will be growing in the inner

layers. If dissolved oxygen concentration is controlled, the undesired presence of nitrite oxidizing organisms will be also avoided.

SBRs for combined partial nitrification/Anammox with continuous feeding and intermittent aeration were implemented on two full-scale plants in Strass (Austria, since 2004) and Glarnerland (Switzerland, since 2007). In these reactors biomass is growing in form of aggregates and they are currently efficiently treating 0.5 g N ($L\ d^{-1}$) (Wett, 2007).

1.7. ECONOMIC ASPECTS OF NITROGEN REMOVAL

1.7.1. Cost analysis of conventional processes

One of the most important factors to design a new treatment plant and to decide what process will be implemented is the estimation of the total cost, including the operational cost along the whole life of the plant.

A recent work by Hunt *et al.* (2009) studied and compared different processes for nutrient removal: Johannesburgo/Bardenpho, modified Ludzack-Ettinger, Step-BNR, Step-Feed and multiple sludge process. All of them are based on the combination of aerobic, anoxic and/or anaerobic stages and on the use of different recirculation streams (Table 1.4). The biological processes are always nitrification and denitrification and the main difference is the configuration of the employed reactors and also the presence or absence of chemical treatments.

The studied volumetric flow per day was 25,000 m^3 and the wastewater to be treated was urban type with 180 mg BOD L^{-1}, 50 mg N_{total} L^{-1} and 6.5 mg P_{total} L^{-1}. It is important to point out that in the case of Step-Feed process, part of the denitrification is carried out with methanol. When the multiple sludge process was considered, all the elimination of nitrogen was carried out with the mentioned external carbon source.

Table 1.4. Evaluated technological alternatives (Hunt *et al.*, 2009).

Technology	Aerobic chambers	Anoxic chambers	Recirculations	Distributed influent
Johannesburg/ Bardenpho	4	2	2	No
Modified Ludzack-Ettinger	4	2	2	No
Step-BNR	3	3	1	Yes (1/3)
Step-Feed	3	1 Denitrifying filter	1	Yes (1/3)
Multiple sludge	2	1 Denitrifying filter	2	No

After the economic evaluation of the five processes, Hunt *et al.* (2009) found out that the most expensive ones were Johannesburg and Ludzack-Ettinger. In both cases the combined cost of installation and operation along 40 years (useful life of the plant) was about 7.6 millions of dollars. Additionally, these both processes were the ones which needed the biggest reaction volume (29,000 m^3), because they used only biological means to remove nutrients.

Multiple sludge process was the one with the smallest reaction volume (11,000 m^3) and, consequently, smallest area. This will be an advantage when the plant is going to be built on very expensive land. However, this is a process with chemical P removal and methanol addition for the N removal, therefore the combined installation and operation cost calculated was almost as high as that mentioned before for the most expensive processes. In this case it was 6.7 M$. The Step-Feed process was about 10% cheaper.

Finally, the most economical process was Step-BNR. In this case total cost along useful life of the plant was 4.8 M$. As a disadvantage, the calculated reaction volume was the second biggest, with 18,000 m^3, which would be important in the case of limited space.

Therefore, according to this work (Hunt *et al.*, 2009) if no space limitation exists the best configuration will be Step-BNR, while if the mentioned limitation is present it can be interesting to use an alternative with at least partial chemical removal of nutrients.

Small treatment plants have some special characteristics, both of employed processes and operation, which make their economical analysis different from the one applied to big plants.

In a recent work, Gallego *et al.* (2008) analyzed the operation of 13 small size treatment plants in Galicia. These plants are operating using three different technologies: long aeration, Biodenipho and aerobic-anoxic treatment. In terms of nutrient removal efficiency, Biodenipho and aerobic-anoxic treatments were the most efficient.

In terms of energy consumption per population equivalent (p.e.; defined as the organic biodegradable equivalent to a BOD_5 of 60 g d^{-1}), the plants with highest values were those with long aeration process, especially the ones operated in a not very rigorous way. The power consumption of those plants was an average of 74 kWh (p.e. year)$^{-1}$. In the case of the plants which were efficiently operated, consumptions reported were much lower: 29 kWh (p.e. year)$^{-1}$. This value is about the maximum reported for treatment plants in Germany (16-29 kWh (p.e. year)$^{-1}$) (MURL NRW, 1999).

One of the main conclusions of the authors (Gallego *et al.*, 2008) is the importance of the operation on the efficiency and the cost, especially for small size plants. Therefore, it would be very important to have rigorous operational procedures, well known and applied by all the plant staff.

Insisting of long aeration technology, broadly used for small size treatment plants, data from the operator of ten urban plants situated in Galicia were analyzed. These plants had treatment capacities between 1200 and 37500 p.e. The operator reported average oxygen consumption

of 0.14 kg $p.e.^{-1}$. This value means about 20-22 kWh $(p.e.\ year)^{-1}$, lower than the previous one reported for long aeration efficiently operated. It is important to consider that in these plants oxygen savings about 15% were reported by including a predenitrification stage to consume part of the biodegradable organic matter. This is an easy modification which would be strongly advisable for small treatment plants.

1.7.2. Comparison of processes based on operational cost

The conventional nitrification/denitrification process and some of the new alternatives will be compared in terms of operational cost along this section. Therefore, the cost to remove 1 kg of NH_4^+-N will be estimated for two different scenarios:

- Wastewater with enough biodegradable organic matter in order to complete denitrification.
- Wastewater with low content of biodegradable organic matter.

It will be assumed that the wastewater to be treated has enough alkalinity, thus carbonate or an alternative chemical will not be necessary. Investment cost to build the plant (very influenced by the land cost) and salaries will not be considered.

The energy to transfer 1 kg of oxygen to clean water is about 0.5-0.9 kWh $(kg\ O_2)^{-1}$ when surface mechanical aerators are employed (WEF and ASCE, 1998). This type of aerators is selected in order to estimate the electric consumption of nitrifying units, because they are very common in wastewater treatment plants. An average of 0.8 kWh $(kg\ O_2)^{-1}$ is chosen as calculation value. The efficiency of the aeration when water contains pollution is about 80% of the oxygen transfer efficiency in clean water (Metcalf and Eddy, 2003). The results of these calculations of oxygen consumption are shown in Table 1.5.

The cost of the electricity is 0.09 € $(kWh)^{-1}$. The external carbon source is methanol because it is the most common one employed. The price of the mass unit of methanol is about 450 \$ t^{-1} in the international market. Assuming an exchange rate about 1.5 \$ $€^{-1}$, the price in Euros will be about 300 € t^{-1}.

Regarding sludge production, the average biomass yield for nitrification/denitrification is about 1 kg VSS (kg N)$^{-1}$ (Fux and Siegrist, 2004). The value is 40% lower for nitrification/denitrification through the nitrite route and about 0.15 kg VSS (kg N)$^{-1}$ for the autotrophic nitrogen removal in one or two steps (Fux and Siegrist, 2004). The percentage of solids (VSS) of the dehydrated sludge was assumed about 22% and the dehydrated sludge treatment cost is about 60€ t^{-1}. With all these data, the total costs per kg of N removed were calculated (Table 1.5).

Table 1.5. Estimation of operational cost to remove 1 kg of NH_4^+-N (oxygen, carbon source and sludge treatment).

Technology	O_2	Electricity		C source		Sludge		Total cost
	kg	kWh	€	kg MeOH	€	kg	€	€
Conventional N/D	4.3	4.3	0.39	-	-	1.0	0.27	**0.66**
Conventional N/D with external C source	4.3	4.3	0.39	2.5	0.75	1.0	0.27	**1.36**
N/D through nitrite	3.4	3.4	0.31	-	-	0.60	0.16	**0.47**
N/D through nitrite with external C source	3.4	3.4	0.31	1.5	0.45	0.60	0.16	**0.92**
Partial N/Anammox (1 or 2 steps)	2.0	2.0	0.18	-	-	0.15	0.04	**0.22**

The obtained operational costs for autotrophic removal in one or two steps are very similar to the one published by van Dongen *et al.* (2001), whose estimation was about 0.22 € (kg NH_4^+-N)$^{-1}$.

Taking into account the obtained results and with conventional nitrification/denitrification as the calculation basis:

- When wastewater contains enough biodegradable COD in order to perform denitrification, nitrification into nitrite allows savings of about 30%. Furthermore, less COD is oxidized during this partial nitrification, so more COD will be available to denitrify. In this case partial nitrification/Anammox will allow 67% of cost savings.

- When wastewater does not contain biodegradable COD to perform denitrification, the potential savings derived from the use of new processes may be significantly higher. The main reason is that in this scenario the cost of the external carbon source (methanol) becomes the most important when denitrification is employed. One additional factor, which is also an advantage of the autotrophic N removal, is the reduced sludge treatment cost. Since Anammox is autotrophic and has low biomass yield, the amount of sludge produced will be significantly smaller than that produced by heterotrophic denitrification.

1.7.3. Modification and improvement of existing plants: actuation on rejected water line

1.7.3.1. Bioaugmentation

As it was previously discussed (Section 1.4.2.2), an interesting technology to improve existing activated sludge plants is the BABE process. It has been used in the treatment plant of Garmerwolde (The Netherlands), which has a capacity of 300000 p.e. (Salem *et al.*, 2004). The implementation of this process at full scale allowed lowering the concentration of ammonium in the effluent from 13.3 to 5.2 mg $N\text{-}NH_4^+$ L^{-1}. This technology was also evaluated in order to improve the operation of Walcheren plant (The Netherlands). Two modifications of the plant were proposed and compared: bioaugmentation and the increase of retention time (larger anoxic and aerobic reaction volumes). It was

demonstrated that the BABE option reduced about 50% the required area, thus the modification cost was about 750,000 € lower. Furthermore, it implied operational cost savings about 11,500 € per year (Salem *et al.*, 2002) (Table 1.6).

Table 1.6. Comparison of two alternatives (increase of units volume and BABE) to improve Walcheren treatment plant.

Unit	Increase of volume of conventional plant	BABE process implementation
	Increase (%) (Additional volume/Original volume)	
Arerobic tank	88	22
Anoxic tank	1,300	370
BABE system	0	14
Total	225	75

1.7.3.2. Anammox process implementation

An interesting alternative in order to improve the nitrogen removal in an existing plant would be to implement the Anammox process. Its application to the rejected water of the plant would allow important savings on energy consumption (Siegrist *et al.*, 2008). The most part of the urban wastewater treatment plants were initially designed only to remove organic matter, thus they were equipped with primary settlers with HRT about 2-3 h in order to reduce the organic load in the biological system. However, the need for elimination of nitrogen caused the HRT of the primary settlers to be reduced to less than 1 h, in order to have enough biodegradable organic matter for the denitrification. If Anammox process is applied to treat the supernatant of the sludge digester, the capacity of the denitrification stage can be reduced without affecting the global nitrogen removal efficiency. This allows increasing back the HRT of the primary settler to improve the biogas production and reducing the aeration energy employed to remove the organic matter.

Taking into account that the aeration energy employed to remove the organic matter and to nitrify is about 70-80% of the total energy consumption of the plant, the new configuration with Anammox process can reduce about 50% of the total energy consumption (Table 1.7).

Table 1.7. Evaluation of energy consumption with the implementation of Anammox process: urban treatment plant; *Case a* without Anammox and primary settler HRT 0.5-1h; *Case b* with Anammox and primary settler HRT 2h (Adapted from Siegrist *et al.*, 2008).

Unit	Mass flow (g (p.e. d)$^{-1}$)		Energy (kWh (p.e. d)$^{-1}$)	
	Case a	Case b	Case a	Case b
COD degradation	40	30	0.040	0.030
N elimination	22	22	0.022	0.022
Pumping and mixing			0.020	0.020
Anaerobic digestion: electricity produced	30	40	-0.038	-0.051
Total			0.044	0.021

1.8. PERSPECTIVES AND FULL SCALE APPLICATIONS OF ANAMMOX

1.8.1. Anammox research topics and trends

Since Anammox was discovered, the specific Anammox research fields and the number of published articles, research projects and, therefore, professionals involved were changing. Like it happens with every new discovery, there was a "lag-phase" during the first years (Figure 1.8) and it took six years to reach an annual number of 10 articles published about Anammox. From this point (2001), an "exponential-like" growth phase took place and the number of annual articles published reached about

100 in only 6 years (2007). The increase of research continued and at the end of 2009 more than 130 Anammox research papers were published.

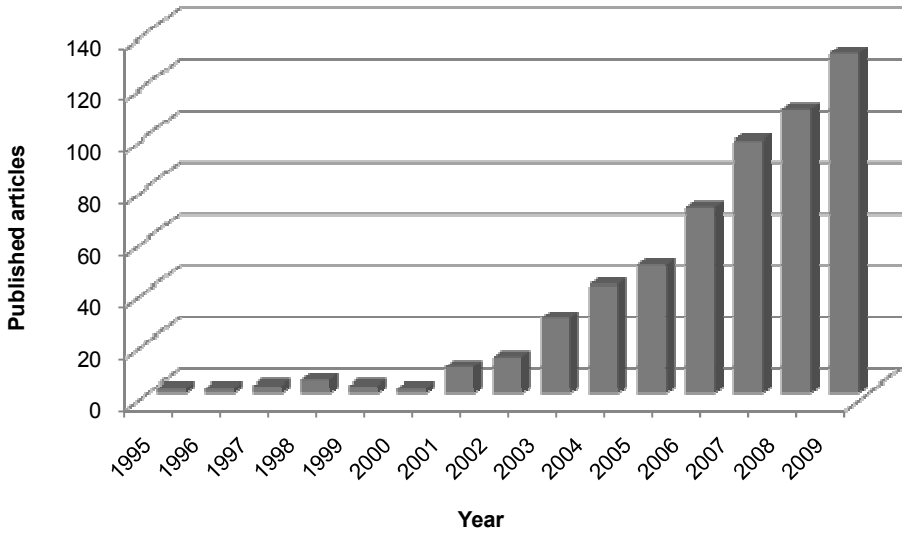

Figure 1.8. Number of Anammox articles published per year (articles indexed on ISI Web of Knowledge with the word "Anammox" in the title or topic).

If specific research topics are examined, during first five years of Anammox publications (1995-1999) the main topics were physiology, metabolism and identification. During the next five years (2000-2004) application strategies, microbiological and microstructure analysis, modeling and reactors were one of the most important issues researched. In this period the first papers about application at pilot and full scale appeared. If a search for articles with the word "Anammox" in the title and "full scale" in the topic is performed, the total number obtained is 14. Only in 2007 and 2009 more than 1 of these articles were published, so full scale application of the process can be considered still in an early stage, despite some plants are already in operation (Table 1.8).

Therefore, it can be assumed that Anammox is now among the most researched topics in wastewater treatment. However, there are still some

limitations and bottlenecks which make the full scale worldwide implementation slower and more difficult. These facts will be discussed along the next section.

1.8.2. Anammox full scale application and perspectives

1.8.2.1. Full scale application

Since Anammox was discovered a number of full scale plants were started up around the world (Table 1.8).

After the very long start up of the first full scale Anammox reactor (Figure 1.9), which took more than three years, the time to start the new reactors was progressively reduced. This improvement has several causes, but the most important is the fact that unlike in the case of the first reactors, enriched Anammox biomass is now available to inoculate new plants. Because of this reason the most recent Anammox systems were started up in few months (Table 1.8).

Most part of the existing full scale plants is treating rejected water from anaerobic digestion in municipal wastewater treatment plants. The advantage of this effluent is its high temperature, that allows to operate the Anammox reactor at good rates.

When attention is focused on the Anammox plants implemented in industrial WWTPs, the first one is treating the effluent of a tannery plant, after biological COD, sulphate and chromium removal (Abma *et al.*, 2007b). The second plant is located in the WWTP of a potato factory. COD removal is performed in an UASB and then phosphate is removed by struvite precipitation (Abma *et al.*, 2007b). Finally nitrogen is removed in the one-stage Anammox reactor. Finally, a two-step Anammox treatment was established in a semiconductor plant in Japan.

Table 1.8. Full scale Anammox plants (Abma *et al.*, 2007a; Abma *et al.*, 2007b; Wett, 2007; Joss *et al.*, 2009; SYVAB, 2009). When the system comprises two sequential units, the presented volume corresponds to the Anammox one.

Project	Application	Volume (m^3)	Capacity achieved (kg N d^{-1})	Start up time (months)
Waterboard Hollandse Delta, Rotterdam, The Netherlands (2 steps)	Municipal (reject water)	72	750	42
Strass, Austria (1 step)	Municipal (reject water)	500	350	30
IndustrieWater Lichtenvoorde, The Netherlands (2 steps)	Tannery	100	150	12
Waterstromen, Olburgen, The Netherlands (1 step)	Potato processing	600	700	6
Himmerfjarden WWTP, Sweden (1 step)	Municipal (reject water)	700	240	6
Glanerland, Switzerland (1 step)	Municipal (reject water)	400	250	2
Semiconductor Plant, Mie prefecture, Japan (2 steps)	Semiconductor	58	220	2
Zürich 1, Switzerland (1 step)	Municipal (reject water)	1400	625	6
Zürich 2, Switzerland (1 step)	Municipal (reject water)	1400	625	0
St. Gallen 1, Switzerland (1 step)	Municipal (reject water)	300	108	7
St. Gallen 2, Switzerland (1 step)	Municipal (reject water)	300	108	5
Niederglatt, Switzerland (1 step)	Municipal (reject water)	160	56	3

Figure 1.9. Anammox full-scale plant in Rotterdam (The Netherlands).

With the progress of the technology and the development of solutions for some of the bottlenecks and limitations detailed along the next section, the implementation of the Anammox process in municipal and, especially, in industrial WWTPs may significantly increase.

1.8.2.2. Anammox perspectives

From the beginning it was realized that the Anammox process had a great potential for the full-scale application in order to remove ammonium from wastewater. In fact, as it was discussed along the previous section, there are some full-size plants which have already been started up (Table 1.8). However there are still some important research issues which should be addressed in order to increase the chances to apply the Anammox process at full-scale.

1.8.2.2.1. High biomass retention

One of the biggest problems for the industrial implementation of the Anammox process are the long start up periods, due to the long duplication time (19 days, (Dapena-Mora *et al.*, 2004)) and low cell yield of

biomass. Furthermore, big amounts of inoculum are not always easily available. Therefore, the crucial aspect is to improve the retention of biomass and minimize its wash out. To achieve this, various solutions have been tried, as the formation of biofilms on a support, the use of SBR reactors or the use of membranes.

Early attempts to improve Anammox biomass retention were conducted using inorganic supports, such as particles of sand (Mulder *et al.*, 1995) in a fluidized bed reactor. Although it succeeded in treating a relatively high NLR (1.8 g N $(L\ d)^{-1}$), the system was not stable. Strous *et al.* (1997b) used a fixed bed reactor with glass spheres as a support to treat a synthetic wastewater and water from a sludge digester. These authors achieved a good retention of biomass and the system was able to efficiently treat a NLR of 1.1 g N $(L\ d)^{-1}$, which is relatively high when compared to traditional systems of denitrification. However, the system became unstable due to retention of nitrogen bubbles inside the bed, causing accumulation of nitrite and inhibition of the activity.

In 1998, Strous *et al.* noted that the sequencing batch reactor was a good alternative for the enrichment of Anammox biomass, reducing the sludge wash-out. Other benefits observed in this system were: the stability of the treatment, since a good distribution of substrates in the reaction mass is achieved, and the easiness of reactor operation. Strous *et al.* (1998) managed to treat a NLR about 1 g N $(L\ d)^{-1}$. Several authors (Van Dongen *et al.*, 2001; Fux *et al.*, 2002; Third *et al.*, 2005) subsequently chose to use SBR reactors to enrich Anammox biomass with good results.

Other options to reduce biomass wash out are the use of membranes for the total retention of the microorganisms within the system (Trigo *et al.*, 2006) or the aggregation of biomass by increase of ionic strength of the medium (i.e. higher saline concentration). This latter strategy minimizes the electrostatic repulsion between cells and allows stronger interactions. (Vázquez-Padín, 2009).

1.8.2.2.2. Presence of possible inhibitors

For the successful implementation of the Anammox process, one aspect is the possible inhibition and its extent in relation with the concentration of the substance in question. This is important because the presence of any of these inhibitors could compromise the Anammox reactor stability. If this destabilization produces the irreversible loss of activity of the biomass or its total wash-out, a new inoculation and start up will be necessary, with the corresponding loss of time and money associated.

As it was pointed out in Section 1.8.2.1, Anammox is very suitable for applications as post-treatment of anaerobic digesters. Actually, many of the full size plants follow this strategy. Therefore, as proposed in this work, effluents from anaerobic treatment of liquid manure would be suitable for the treatment with the Anammox process, presenting very low C/N ratios and high nitrogen loads. However because of the origin of these wastewaters, the presence of significant concentrations of pharmaceutical veterinary compounds can be expected, which may affect the efficiency of the process. Among these compounds, antibiotics are one of the most important groups, because of the amounts consumed per year. Actually, the typical concentrations of antibiotics found in slurries are relatively high. For example, Huang *et al.* (2001) found concentrations of tetracycline, sulfathiazole and penicillin above 100 mg kg^{-1} in sheep manure.

The effects of antibiotics on the anaerobic digestion of manure have been researched (Massé *et al.*, 2000; Chelliapan *et al.*, 2006), but there is no information about if they can affect the Anammox post-treatment. Therefore this information can be a key factor in order to achieve the implementation of Anammox process to treat the effluents of anaerobic digestion of manure.

Moreover, as noted above (section 1.5.5.3), Anammox suffers inhibition by substrate (nitrite and ammonium). Therefore, substrate ratios far from

the stoichiometric one can lead to the loss of the activity. This fact will be more likely to happen during the start up of the partial nitrification stage when ammonium to nitrite ratio is still not very well controlled. When the Anammox is implemented as a one stage process, the problem can also occur during its start up. Taking into account the difficulties for the start up of Anammox reactors and the chance to need very long times to achieve the design NLR removal, episodes of inhibition by substrates are strongly undesirable. Therefore, it would be very interesting to have the inhibitory effects and the concentration thresholds for each of the substrates well established.

1.8.2.2.3. Application at mesophilic temperature

Operation at relatively high temperatures ensures a good Anammox activity (section 1.5.5.1) and almost all full scale Anammox reactors are operated at a temperature near to 30 ºC. Heating is not necessary since these Anammox reactors are following anaerobic digesters, which are themselves operated at relatively high temperature (usually mesophillic range). Therefore, the influent fed to the Anammox system is already at the appropriate temperature.

However there are wastewaters which can be potentially treated by Anammox, but which are at relatively low temperature. A deeper knowledge about the performance of the Anammox process at relatively low temperatures can significantly widen the types of wastewaters which can be treated, increasing the opportunities to implement full scale Anammox plants.

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Chapter 2

Materials and methods

In this chapter, the materials and methods employed to carry out the experimental work of this Thesis are explained. First, the methods employed to analyze the properties of the liquid phase are detailed. They are followed by the techniques for the physical and microbiological characterization of the biomass. Finally, the composition of the Anammox mineral medium is included. This medium was used to prepare the synthetic Anammox feeding media.

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

2.1. LIQUID PHASE

In this section, the methods used for the determination of the conventional parameters of water 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, except when samples came from reactors with biofilm biomass on inorganic carriers (i.e. zeolites). In this latter case the filtration was using a pore size of 0.22 μm.

2.1.1. Ammonium-nitrogen

Ammonium nitrogen was 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 at 635 nm.

2.1.1.1. Reagents

Solution 1: Phenol-nitroprusiate: 15 g of phenol and 0.05 g of sodium nitroprusiate were 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 litre, adjusted to pH 12.

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

2.1.1.2. Procedure

The volume of sample was 2.5 mL (diluted if necessary to get a maximum concentration of 1 mg NH_4^+-N L^{-1}). 1.0 and 1.5 mL of solution 1 and 2 were added to the sample. After 45 min at room temperature, the intensity of colour was measured in a spectrophotometer (Shimadzu UV-1603 or Cecil Instruments Aquarius CE 7200) at 635 nm. The quantification was done with a 5-7 points calibration curve in the range of 0-1 mg NH_4^+-N L^{-1}, using a commercial standard solution of NH_4^+ (1000 mg NH_4^+ L^{-1}; CertiPUR, Merck) (Figure 2.1).

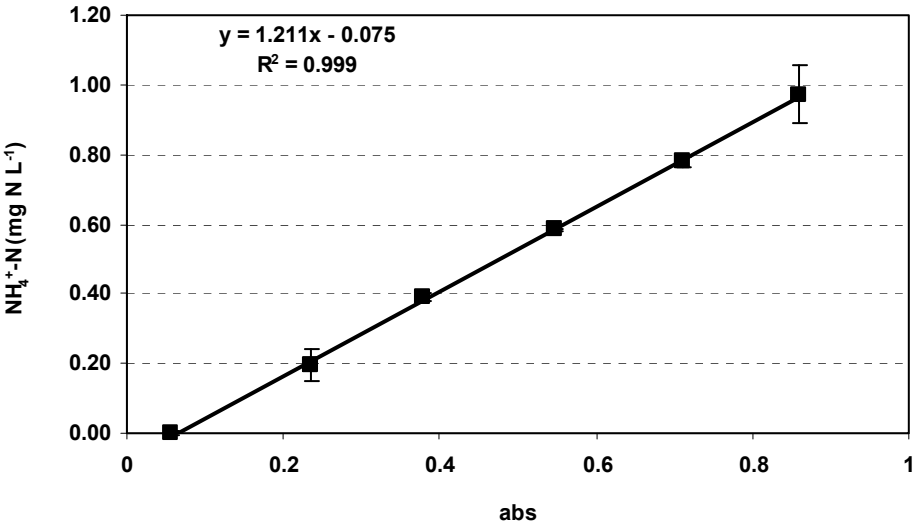

Figure 2.1. Calibration curve for ammonium concentration determination.

2.1.2. Nitrite-nitrogen

Nitrite concentration was determined following the method 4500-NO_2^- B described in Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 1998). Nitrite was determined through the formation of a reddish purple azo dye produced at pH 2.0-2.5 by coupling diazotized sulphanilamide with N-(1-napththyl)-ethylenediamine dihydrochloride (NED dihydrochloride).

2.1.2.1. Reagents

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

Solution 2: NED: 0.5 g of NED were dissolved in 500 mL of distilled water.

2.1.2.2. Procedure

0.1 mL of each solution were added to 5 mL of sample (diluted if necessary to have a maximum concentration of 0.30 mg NO_2^--N L^{-1}). After

20 min, the intensity of colour of the sample was measured in a spectrophotometer (Shimadzu UV-1603 or Cecil Instruments Aquarius CE 7200) at 543 nm. The quantification was done with a 5-7 points calibration curve in the range of 0-0.30 mg NO_2^--N L^{-1}, using a commercial standard solution of NO_2^- (1000 mg NO_2^- L^{-1}; CertiPUR, Merck) (Figure 2.2).

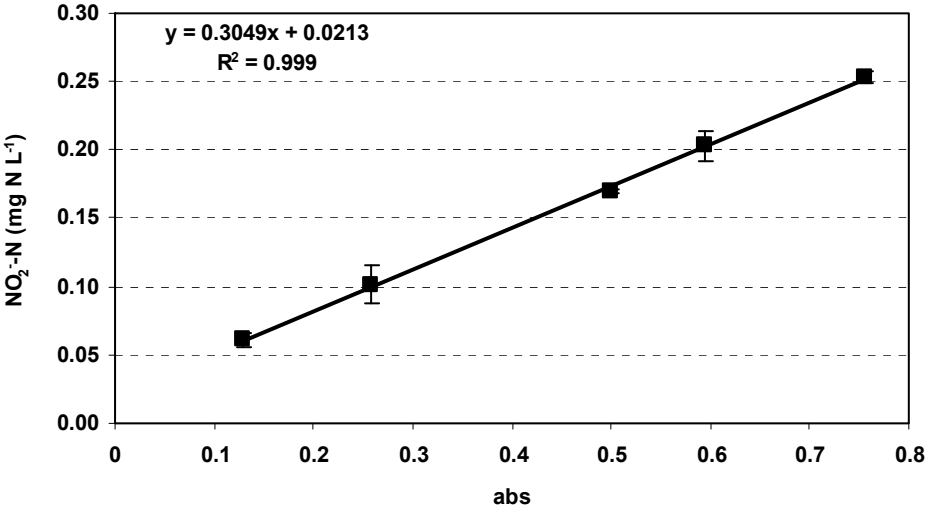

Figure 2.2. Calibration curve for nitrite concentration determination.

2.1.3. Nitrate-nitrogen

Nitrate concentration was determined following the method 4500-NO_3^- B described in Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 1998). Measurement of UV absorption at 220 nm allowed the fast determination of NO_3^- ions. Since dissolved organic matter may also absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement at 275 nm was used to correct the NO_3^- value.

2.1.3.1. Procedure

5 mL of sample (diluted if necessary to get a maximum concentration of NO_3^--N of 2.5 mg L^{-1}) were taken. 0.1 mL of HCl 1N were added to each

sample. Afterwards, the absorbance at 220 and 275 nm was measured in a spectrophotometer (Shimadzu UV-1603 or Cecil Instruments Aquarius CE 7200). The absorbance related to nitrate was obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm. The quantification was done with a 6-8 points calibration curve in the range of 0-2.50 mg NO_3^--N L^{-1}, using a commercial standard solution of NO_3^- (1000 mg NO_3^- L^{-1}; CertiPUR, Merck) (Figure 2.3).

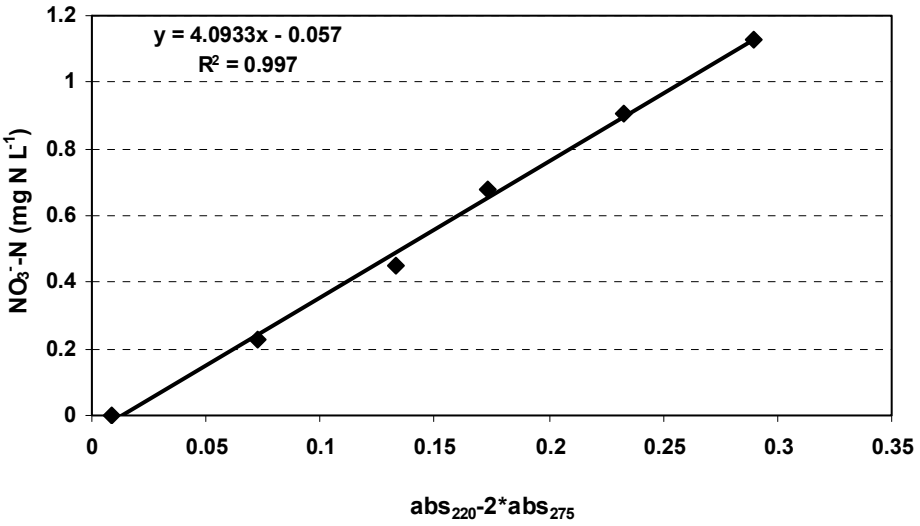

Figure 2.3. Calibration curve for nitrate concentration determination.

2.1.4. Inorganic anions determination by capillary electrophoresis: NO_2^-, NO_3^-

Nitrite (NO_2^-) and nitrate (NO_3^-) were determined simultaneously by Waters Capillary Ion Analyzer (CIA). A solution of sulphate was used as electrolyte (Vilas-Cruz *et al.*, 1994). An electro-osmotic modifier CIA-PakTM OFM Anion BT Waters (Ewing *et al.*, 1989) was also added to this electrolyte solution. The sample was forced to migrate through a capillary (melting silica covered with poliimida, 60 cm long and 75 µm of internal diameter) by the application of an electric current (20 kV, 36-40 µA). Temperature of the capillary was maintained at 25 ºC. Depending on the ratio

charge/mass of the ion, the migrating time is different. A hydrostatic injection (10 cm height for 4 seconds) and direct detection (UV, 214 nm) were used. A number of 4 to 6 calibration points for each ion were daily used for the quantification of the samples.

2.1.5. pH

The pH measurements were performed with an electrode (Crison Instruments, 52-03) connected to a measure instrument (pH mV^{-1}) Crison GLP 21 or GLP 22. The sensibility of the system was ±1 mV, corresponding to 0.01 pH units. The electrode was calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

2.2. BIOMASS CHARACTERISATION

The methods employed to measure the physical properties of the biomass are detailed along this section.

2.2.1. Total and Suspended Solids

Total Suspended Solids (TSS) Volatile Suspended Solids (VSS) and inorganic suspended solids were determined according to the methods 2540D and 2540E described in Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 1998).

2.2.1.1. Procedure

For the determination of TSS, a selected (in order to yield a residue between 2.5 and 200 mg) well-mixed sample volume was filtered through a weighed glass-fiber filter (Whatman, GF/C, 4.7 cm of diameter, 1.2 µm of pore size) and the residue retained on the filter was dried to a constant weight (2h) at 103-105 ºC. The increase in weight of the filter represents the TSS.

To determine the VSS, the residue from method 2540D was burnt to constant weight at 550 ºC during half an hour. The weight lost during the

ignition corresponds to the volatile solids, since only a very small amount of inorganic salts are decomposed and volatilised at that temperature.

2.2.2. Sludge Volume Index

The Sludge Volume Index (SVI) determination is defined in the Standard Methods for the Treatment of Water and Wastewater (APHA-AWWA-WPCF, 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 consolidates much faster, i.e., the terminal SVI_{30} is already reached after 5 minutes of settling.

2.2.3. Granular biomass density

The biomass density (expressed as mass of granules per volume of granules) was determined using the method described by Beun *et al.* (2002). Firstly, a known amount of a homogeneous biomass sample was taken from the reactor. Then, a measured amount of liquid was removed from the sample. A known volume of a dextran blue solution (1 g L^{-1}) was added to the sample, in a volume ratio of about 1:1. The mixture is gently mixed, and subsequently the granules are allowed to settle. Some amount of the liquid above the settled granules is taken. This fraction and the original dextran blue solution are analyzed by a spectrophotometer (Shimadzu UV-1603 or Cecil Instruments Aquarius CE 7200) 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 Equation 2.1 and V_L with Equation 2.2.

$$Density = \frac{V_{initial} x VSS}{V_{biomass}} \quad (2.1)$$

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

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

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

P_1: test tube weight (g)

P_2: weight of the test tube with sample (g)

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

P_4: weight of the test tube after dextran blue addition (g)

ρ_w: density of water (g L^{-1})

$$V_L = \frac{A_{initial} x V_{dextran}}{A_{final}} \quad (2.2)$$

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

A_{final}: Absorbance of the sample

$V_{dextran} = (P_4 - P_3)/\rho_w$ (L)

2.2.4. Average diameter of the granules

Biomass samples were observed with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (Stemi 2000-C, Zeiss). Furthermore, the same digital imaging system was employed to monitor changes in morphology of the granular biomass by image analysis (Tijhuis *et al.*, 1994). Images of the granular sludge were taken and least 100 granules were photographed per sample. The programme Image ProPlus® was later employed for the digital image analysis.

The image analysis procedure employed is as follows:

I) Convert the original photo of granules to black and white mode, since it simplifies the image processing.

II) Select the measurements of interest. Usually they were: area, roundness, and Feret diameters (minimum, maximum and average). Feret diameter is the distance between two tangents on opposite sides of the granule.

III) Set the minimum particle size to be considered as a granule. In this work this limit was 0.1 mm.

IV) Define the range of colours corresponding to the area of interest in the image, i.e. the granules. This can be done automatically or manually.

V) Count the granules, split the ones which were measured together and discard the particles which are not granules.

VI) Export the data of interest to a worksheet.

The average diameter obtained from the programme corresponded to the mean Feret diameter of the granules. The mean Feret diameter is calculated as an average value from the shortest and the longest measured segment in the granule (Figure 2.4).

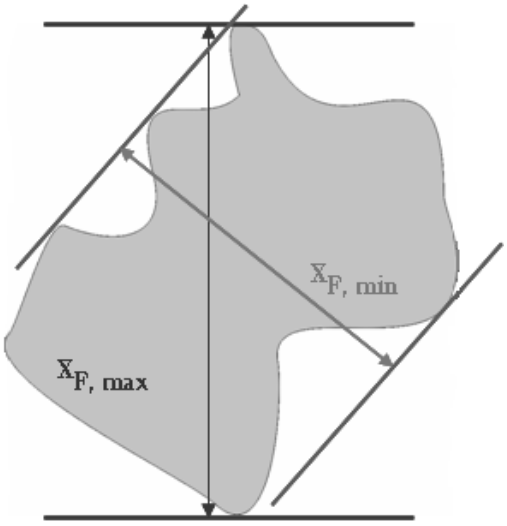

Figure 2.4. Longest and shortest segments (i.e. minimum and maximum Feret) in a granule to estimate its mean Feret diameter.

2.2.5. Specific Anammox activity assays

The batch assays used to measure the maximum Specific Anammox Activity (SAA) were performed according to the methodology described by Dapena-Mora *et al.* (2007).

Completely closed vials with a total volume of 38 mL and 25 mL of liquid volume were used to perform the Anammox batch assays. The procedure was as follows:

I) 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.

II) Gas and liquid phases were purged with an inert gas (Ar, He) to remove O_2.

III) The vials were placed in a thermostatic shaker, at 150 rpm and the temperature T.

IV) After some minutes for thermal stabilization, substrates were added into the vials. Initial concentrations of substrates were 70 mg NH_4^+-N L^{-1} and 70 mg NO_2^--N L^{-1}, except when inhibition by substrates was researched. In that case, different concentrations were used, as it is detailed in Chapter 3.

V) The production of N_2 was measured (pressure transducer Centrepoint Electronics) in the gas phase as the increment of pressure in the headspace of the vials.

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. 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.

2.3. MICROBIOLOGICAL DETERMINATIONS

2.3.1. Identification of bacterial populations by FISH

The different populations of microorganisms present in the sludge samples of the reactors were 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 (Figure 2.5): 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. 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).

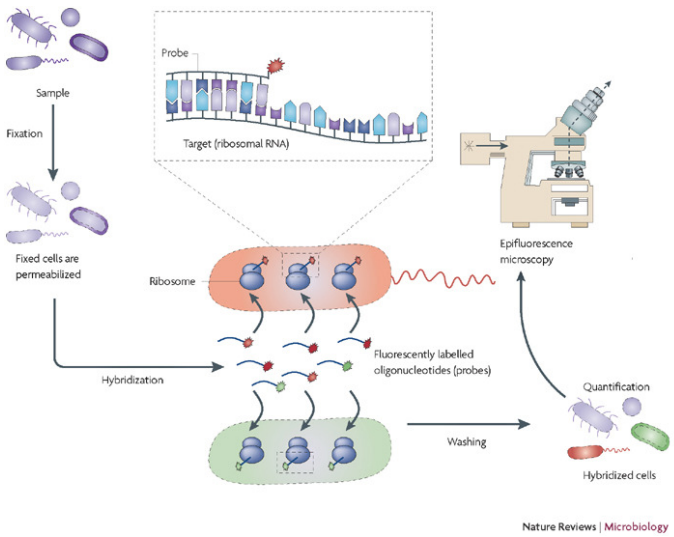

Figure 2.5. Basic steps of FISH technique. (Adapted from Amann and Fuchs, 2008).

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 was minimized by fixation in freshly prepared (less than 24 h) 4% paraformaldehyde solution in PBS. After fixation, the cells were immobilized on a microscopic slide and used for hybridization with 16S rRNA probes. In order to avoid non-specific binding of the rRNA probes, the hybridization was done at stringent conditions (46 ºC, 0-65% formamide) and specimens were washed with buffer (48 ºC). The targeted organisms were 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. For analysis of the slides an epifluorescence microscope (Axioskop2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics) was used. The probes applied are listed and detailed in each chapter.

2.4. MINERAL MEDIUM

Table 2.1 shows the composition of the Anammox mineral medium and traces solution (adapted from Dapena-Mora *et al.*, 2004). This medium was used as base to prepare the different synthetic feedings treated by some of the reactors employed in this Thesis, as it is detailed in the Materials and methods section of each chapter.

Table 2.1. Anammox mineral medium and traces solution composition.

Mineral medium		Traces solution	
Compound	Conc. (mg L^{-1})	Compound	Conc. (g L^{-1})
NH_4^+-N	Variable	EDTA	15
NO_2^--N	Variable	$ZnSO_4 \cdot 7\ H_2O$	0.43
$KHCO_3$	1.25	$CoCl_2 \cdot 6\ H_2O$	0.24
$CaCl_2$	1.41	$MnCl_2 \cdot 4\ H_2O$	0.99
KH_2PO_4	50	$CuSO_4 \cdot 5\ H_2O$	0.25
$MgSO_4$	58.6	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.22
$FeSO_4 \cdot 7H_2O$	9.08	$NiCl_2 \cdot 6H_2O$	0.20
EDTA	6.25	$NaSeO_4 \cdot 10H_2O$	0.20
		H_3BO_3	0.014
Traces solution	1.25 mL L^{-1}	$NaWO_4 \cdot 2H_2O$	0.05

2.5. REFERENCES

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Chapter 3

Short- and long-term effects of substrates on the Anammox process

ABSTRACT. Ammonia and nitrite can exert inhibitory effects on Anammox organisms, causing the decrease of the specific biomass activity and the loss of the operational stability. The aim of the present work is to evaluate these effects in short- and long-term experiments.

The short-term effects were studied with two types of Anammox biomass: biofilm growing on inorganic carriers and flocculent sludge. The results indicated a decrease of the Specific Anammox Activity (SAA) of 45% at about 30 mg NH_3-N L^{-1}, while 100 mg NH_3-N L^{-1} caused an inhibition of 80%, without significant differences between both kinds of biomass. In the case of the biofilm, the SAA was not affected at concentrations up to 6.6 µg HNO_2-N L^{-1}, but suffered a decrease over 70% in presence of 13 µg HNO_2-N L^{-1}. The flocculent biomass was much less resistant and its SAA sharply decreased below 30% in the presence of 4.4 µg HNO_2-N L^{-1}.

The study of the long-term effects was carried out in lab-scale Sequencing Batch Reactors (SBR) inoculated with the Anammox biofilm biomass. Concentrations up to 20 mg NH_3-N L^{-1} showed no effects. However, when free ammonia concentrations reached values between 35-40 mg NH_3-N L^{-1}, the operation became unstable. This destabilization was also observed at nitrous acid concentrations around 1.5 µg HNO_2-N L^{-1}. Total restoration of the system efficiency was achieved after the stoichiometric feeding was applied to the reactor.

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[1] Some parts of this chapter have been published as: **Fernández I., Dosta J., Fajardo C., Mosquera-Corral A., Campos J.L., and Méndez R.** Short- and long-term effects of ammonium and nitrite on the Anammox process. *Journal of Environmental Management*. In press.
Selected results were also presented as: **Fernández I., Dosta J., Campos J.L., Mosquera-Corral A. and Méndez R.** Short- and long-term effects of ammonia and nitrite on the Anammox process. Third International Meeting on Environmental Biotechnology and Engineering. Palma de Mallorca, Spain, September 2008.

3.1. INTRODUCTION

In order to achieve the successful operation of the Anammox process, the potentially negative effects of the compounds present in the wastewater should be considered. Among these compounds, the substrates of Anammox process (nitrite and ammonium) were reported to be responsible for loses in the Anammox activity (Strous, 2000; Dapena-Mora *et al.*, 2007). Taking into account that during start up or overload periods both substrates could not be completely consumed, their presence in the reaction medium should cause the decrease of biomass activity and destabilization of the reactor. These negative effects must be avoided since a new start-up or the recovery of the biomass of the reactor may take a long time, especially in the case of industrial-size reactors (van der Star *et al.*, 2007), due to the very slow growth rate of Anammox bacteria (Strous *et al.*, 1999).

Some studies reported data about the inhibitory effect of ammonia and nitrite (Strous, 2000; Fux *et al.*, 2004; Jetten *et al.*, 2005; Dapena-Mora *et al.*, 2007). However, these works were sometimes carried out under very different operational conditions (pH, temperature, continuous/batch tests…) which entails that the results are difficult to extrapolate and to use for the efficient operation of the reactors. Moreover, different works about the effects of nitrite did not agree about the concentration threshold that would not be exceeded. In the literature different ranges for this threshold (50-150 mg NO_2^--N L^{-1}, Strous *et al.*, 1999; 30-50 mg NO_2^--N L^{-1}, Fux *et al.*, 2004) can be found. The knowledge about safe levels would be very important for the operators of the wastewater treatment plants with Anammox stages in order to maintain the performance of the system.

Therefore, it will be very useful to determine the short- and long-term effects of nitrite and ammonia on the Anammox process in order to apply these results to maintain the performance of the reactors. The unionized

compounds (free ammonia (FA) and free nitrous acid (FNA)) are well known as responsible for the inhibition of both, ammonia- and nitrite-oxidizing bacteria (Anthonisen *et al.*, 1976; Vadivelu *et al.*, 2007; Park and Bae, 2009). Free nitrous acid has also been reported as inhibitor for poly-phosphate accumulating denitrifiers (Zhou *et al.*, 2007). Taken all this into account, these unionized compounds may be assumed to be responsible for the inhibition of Anammox process (Tang *et al.*, 2009). An additional advantage of using inhibition threshold concentration levels in terms of the unionized compounds is that their use in the control of different Anammox systems with varied conditions may be easier.

3.2. OBJECTIVES

The first objective of this work was to evaluate the short-term effects of free ammonia and free nitrous acid on the Anammox activity. For this purpose, Specific Anammox Activity batch tests were employed. The possible influences of the type of counterion and biomass were also researched.

The second objective was to evaluate the long-term effects of the unionized compounds To achieve this aim, an Anammox reactor was operated in presence of different concentrations of both ammonia and nitrite. The study was focused on the capacity of the treatment, its efficiency and the properties of the biomass.

3.3. MATERIALS AND METHODS

3.3.1. Anammox inhibition tests

To determine the short-term effects of FA and FNA on the Anammox biomass and to monitor the operation of the reactors, batch experiments were performed according to the methodology described in Section 2.2.5 (Dapena-Mora *et al.,* 2007). Two types of biomass were employed: biofilm

biomass taken from SBR1 and SBR2 (Sections 3.3.2 and 3.3.4) and flocculent biomass (Dapena-Mora *et al.*, 2004a) with a SAA of 0.10 g N (g VSS d)$^{-1}$. The concentrations of substrates employed for the FA inhibition tests were 70 mg NO_2^--N L^{-1} and ammonium concentrations of 70, 700, 1400, 2100 and 2800 mg NH_4^+-N L^{-1}. Ammonium was supplied as ammonium chloride and ammonium sulphate, in order to research the effect of the counterion. When the inhibition by FNA was studied, the concentrations of substrates were a fixed initial ammonium concentration of 70 mg NH_4^+-N L^{-1} and nitrite concentrations of 70, 140, 210, 280, 350 and 420 mg NO_2^--N L^{-1}.

3.3.2. Experimental set-up

Experiments were carried out in two Sequencing Batch Reactors of 5 L (SBR1) and 3 L (SBR2) of useful volumes, respectively. Temperature was controlled at 33 ºC (SBR1) and 30 ºC (SBR2) by using thermostatic jackets. The complete mixture inside both reactors was achieved using mechanical stirrers with rotating speed of 150 rpm. The control of the pumps and different periods of the operational cycles was performed using a PLC system (CPU224, Siemens). Both reactors were operated in cycles of 6 hours distributed in four periods: mixed fill (300 min), mix (30 min), settle (15 min) and draw (15 min) according to Dapena-Mora *et al.* (2004b). The exchange volume was fixed at 25% and the Hydraulic Retention Time (HRT) was maintained at 1 day in both cases.

To prevent the oxidation of the excess of nitrite to nitrate by oxygen transference, the headspace of SBR2 was continuously flushed with 200 mL min^{-1} of Ar.

3.3.3. Feeding media and operational strategy

Both reactors were fed with a synthetic autotrophic medium described in Section 2.4 (Dapena-Mora *et al.,* 2004a). The strategy in both cases was to fix an initial ammonium to nitrite molar ratio approximately equal to the stoichiometric value (1.32 NO_2^--N/NH_4^+-N according to Strous (2000)).

Then the concentrations of ammonium (SBR1) or nitrite (SBR2) were stepwise increased (Table 3.1). In the case of SBR2, during a first period (designed in Table 3.1 as Previous Period) nitrite concentration was increased from 150 mg NO_2^--N L^{-1} to 225 mg NO_2^--N L^{-1}. This strategy was unsuccessful because no nitrite accumulation was achieved in the reaction medium due to its oxidation into nitrate. After this period, argon was flushed in the headspace of the reactor and the oxidation was completely prevented. Therefore day 0 for this reactor corresponds to the first day with Ar flushing and stoichiometric substrates ratio (Table 3.1)

Table 3.1. Operational strategy.

Reactor	Periods	Days	NH_4^+−N_{inf} (mg L^{-1})	NO_2^-−N_{inf} (mg L^{-1})
SBR1	I	0-25	180	250
	II	26-39	250	250
	III	40-74	375	250
	IV	75-103	425	250
	V	104-172	500	250
	VI	173-200	750	250
SBR2	Prev.	(67)	150	150→225
	I	0-48	150	185
	II	49-69	150	200
	III	70-95	150	220
	IV	96-118	150	240
		119-132	150	200
	V	133-160	150	240
		161-166	150	280
		167-221	150	200
	VI	222-257	150	300
	VII	258-281	150	400

Note: Shaded cells correspond to instability and recovery periods.

Effective Nitrogen Loading Rate (NLR) will be calculated from the concentration of limiting substrate in the feeding plus the stoichiometric concentration of the substrate in excess. The sum of these two concentrations divided by the hydraulic retention time will give the value of the Effective NLR. The system would not be able to remove more

nitrogen than that given by this Effective NLR, since the surplus of the substrate in excess cannot be consumed.

3.3.4. Biomass characteristics

SBR1 was inoculated with enriched Anammox biofilm sludge from a laboratory scale SBR operated at the University of Santiago de Compostela (Figure 3.1; Fernández *et al.*, 2008). SBR2 was inoculated with the biomass of SBR1 at the end of its operation. The initial concentrations of biomass were 1.2 and 2.0 g VSS L^{-1} for SBR1 and SBR2, respectively. The initial SAA values were 0.49 g N (g VSS $d)^{-1}$ and 0.21 g N (g VSS $d)^{-1}$ for the biomass of SBR1 and SBR2, respectively. The support of the biomass was zeolite (ZeoCat, Spain) with a 96% degree of purity, nominal ammonium adsorption capacity between 22.4 and 30.8 mg NH_4^+–N (g zeolite)$^{-1}$ and particle size between 0.5 and 1.0 mm (sieved).

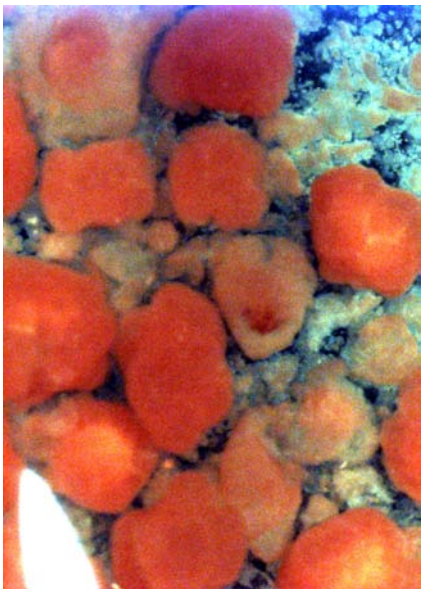

Figure 3.1. Stereomicroscope image of the inoculum of SBR1 at the beginning of the operation.

3.3.5. Nitrifying activity tests

Since nitrite accumulation in the reactor was not achieved during the first attempt (Section 3.3.3) and the oxidation of nitrite into nitrate was

suspected to be taking place, activity tests were performed in order to measure the nitrifying capability of the biomass.

The assays were carried out by means of a respirometric method (adapted from López-Fiuza *et al.*, 2002) based on measurements of the oxygen concentration along time. These tests were performed using a Biological Oxygen Monitor (BOM, YSI model 5300) with oxygen selective electrodes (YSI 5331) connected to a data acquisition system (Figure 3.2). This system is a discontinuous respirometer that uses 15 mL vials with a maximum useful volume of 10 mL, however in the present work, the employed volume was 5 mL in each vial.

Figure 3.2. Respirometry system: left, thermostatic test cells; centre, biological oxygen monitor; right, data acquisition system.

The biomass was washed with phosphate buffer (1.43 g L^{-1} KH_2PO_4, 7.47 g L^{-1} K_2HPO_4). 5 mL of biomass resuspended in buffer medium was added to each vial, and vials were placed in a thermostatically controlled chamber at 30 ºC. Compressed air was used to obtain the initial level of oxygen saturation in the liquid medium. The two electrodes for oxygen measurement were calibrated. Then, the electrodes were inserted into the

vials carefully, in order to avoid the presence of bubbles in the liquid, and the data acquisition software was initialized. After two minutes of oxygen consumption in absence of substrate (endogenous phase), the required substrate was injected in order to achieve a concentration of 70 mg N L^{-1}. This caused faster oxygen depletion, reflected by a steeper slope. After 10 minutes, the test was finished and the biomass concentration in each vial was determined. With the measured oxygen consumption rate, the specific activity was calculated as the substrate consumption rate divided by the biomass concentration.

3.3.6. FISH

Microbial populations were monitored by the Fluorescence In Situ Hybridization (FISH) technique. Biomass samples were collected, disrupted and fixed, according to the procedure described by Amann *et al.* (1995), with 4% paraformaldehyde solution. Hybridization was performed at 46 ºC for 90 minutes adjusting formamide concentrations at the percentages shown in Table 3.2. The used probes for in situ hybridization were labelled with the fluorochromes FITC and Cy3. Fluorescence signals of disrupted samples were recorded with an acquisition system coupled with an Axioskop 2 epifluorescence microscope (Zeiss).

Table 3.2. FISH probes.

Probe	Sequence (5' → 3')	% Formamide	Reference
EUB338-I	GTC GCC TCC CGT AGG AGT	35	Amann *et al.*, 1990
EUB338-II	GCA GCC ACC CGT AGG TGT	60	Daims *et al.*, 1999
Pla46	GAC TTG CAT GCC TAA TCC	20	Neef *et al.*, 1998
Amx820	AAA ACC CCT CTA CTT AGT GCC C	35	Schmid *et al.*, 2001
Kst1275	GTT CCG ATT GCT CGA AAC	25	Schmid *et al.*, 2001
Ban162	CGG TAG CCC CAA TTG CTT	40	Schmid *et al.*, 2001

3.4. RESULTS AND DISCUSSION

3.4.1. Short-term effects of ammonia and nitrite

In order to assess the short-term effects of ammonium, three series of batch assays were performed, two employing biofilm biomass and one with flocculent biomass (Section 3.3.1). Results obtained are presented on basis of the Free Ammonia (FA) concentration, calculated according to Anthonisen *et al.* (1976), due to the fact that unionized ammonia is considered to be the true inhibitor (Figure 3.3).

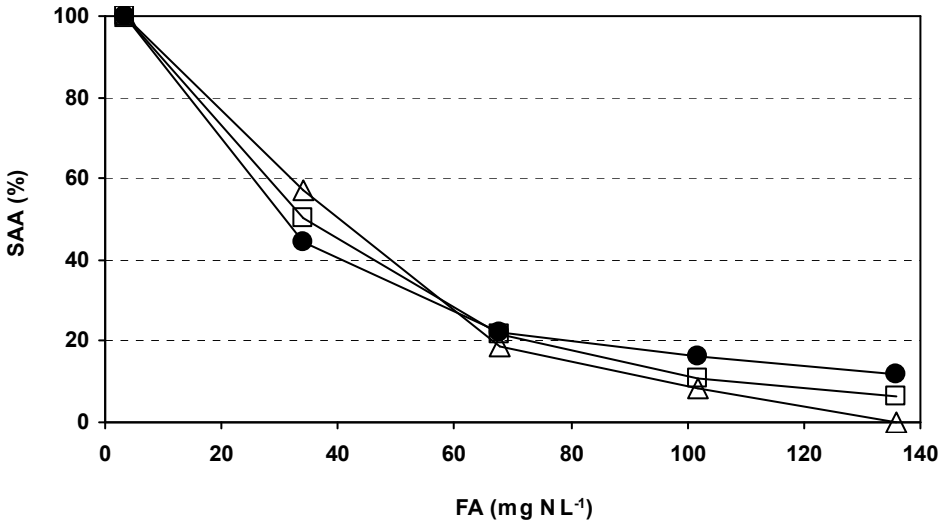

Figure 3.3. Short-term effects of FA (□ biofilm biomass, ammonium chloride; Δ biofilm biomass, ammonium sulphate; ● flocculent biomass, ammonium chloride).

Tests were performed both, with ammonium chlorate and sulphate, in order to evaluate a possible effect of the counterion, however this hypothesis was not confirmed. According to Figure 3.3, FA concentrations higher than 35 mg NH_3-N L^{-1} produced a loss in the SAA larger than 50%.

Since no effect of the counterion was observed when the biofilm biomass was tested, the assays with flocculent biomass were only performed with

ammonium chloride. The SAA profile obtained was equivalent to the ones calculated for the biofilm biomass, therefore no effect of the type of biomass was observed.

Two series of batch tests were performed to evaluate the effects of nitrite on the two kinds of biomass (Section 3.3.1). The chemical specie that was assumed to be responsible for the observed inhibitory effects is the Free Nitrous Acid (FNA). Therefore, the results of the tests are presented in function of HNO_2 concentration (Figure 3.4), calculated according to Anthonisen *et al.* (1976).

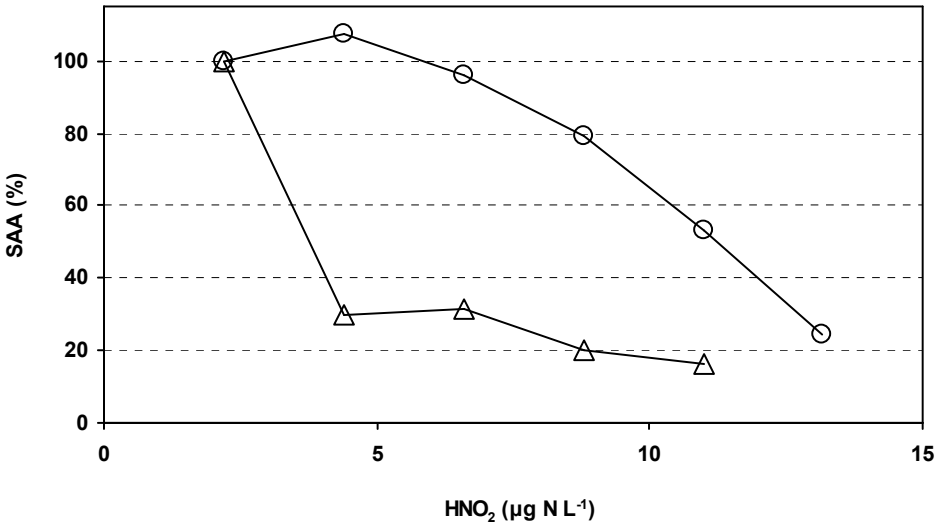

Figure 3.4. Short-term effects of FNA (○ biofilm biomass; Δ flocculent biomass).

In this case, no significant loss of SAA was observed up to 6.6 µg HNO_2-N L^{-1} when biofilm biomass was employed. The 50% inhibition concentration for this kind of biomass (IC50) was detected at 11 µg HNO_2-N L^{-1}. The flocculent biomass was much more seriously affected by the FNA; an inhibition of 70% was found at only 4.4 µg HNO_2-N L^{-1}. SAA was not measurable over 11 µg HNO_2-N L^{-1}. There are two possible factors which

could lead to these differences between both types of biomass. The first one is that zeolites acting as a support for the biofilm are able to adsorb ammonium (Fernández *et al.*, 2008). Therefore, if they have some amount of this substrate adsorbed, they may act as a reserve of ammonium, partially balancing the substrate ratio and reducing the effective excess of nitrite. The second factor is that biofims are considered to be more resistant to changes in the environmental conditions (temperature, pH, toxics) because the outer layers can protect the inner layers. This stronger resistance has been observed more than 20 years ago in experiments with nitrifying biofilm (Olem and Unz, 1980) and ferro-oxidizing biofilm (Karamanev and Nikolov, 1988).

3.4.2. Long-term effects of free ammonia

The reactor SBR1 was operated for 200 days. During Period I the reactor was fed with a stoichiometric ammonium to nitrite molar ratio and the presence of FA in the media was negligible (Figure 3.5). From day 26 the concentration of ammonium was increased stepwise along the different operational periods. During Periods II and III the average ammonium concentrations were 40 and 92 mg NH_4^+-N L^{-1}, respectively. However, in both periods FA concentrations were around 4 mg N L^{-1} due to a decrease of the pH value in the reactor, which favours the displacement of the equilibrium to the ionized specie. From day 75, pH was maintained at a value around 7.8 and FA concentration inside the reactor increased up to 7 and 15 mg NH_3-N L^{-1} (average values) during Periods IV and V, respectively, according to the increase of ammonium concentration. During the first five periods of the operation, the efficiency of the reactor in terms of limiting substrate (nitrite) consumption was very close to 100% (Figure 3.6). However in the Period VI, when the FA concentration was in the range of 35-40 mg N L^{-1}, the operation became unstable. On day 200 of operation, when the efficiency had dropped to around 40%, the experiment was stopped.

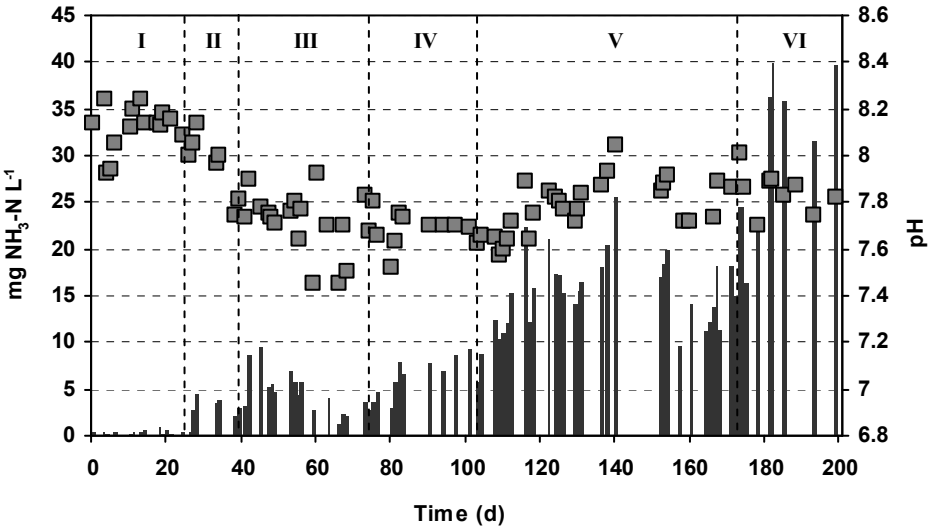

Figure 3.5. FA concentrations (bars) and pH (■) in SBR1.

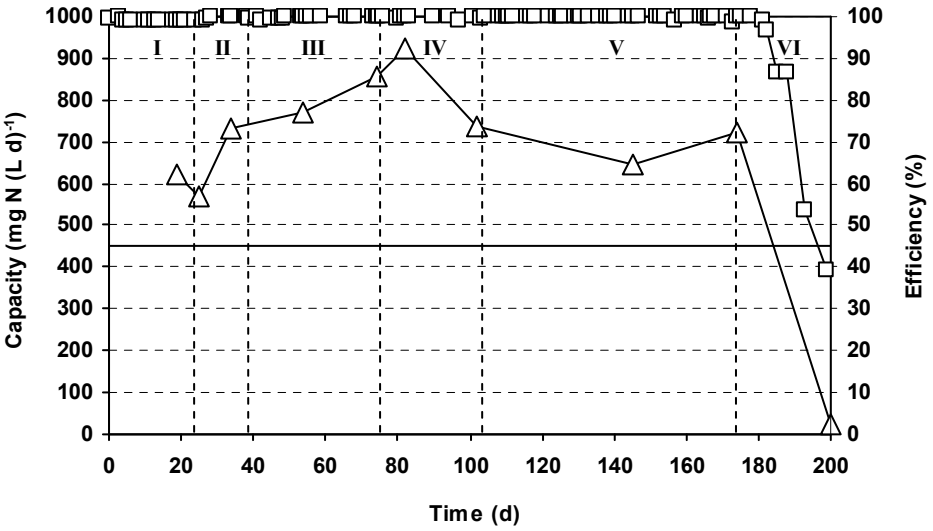

Figure 3.6. Efficiency (□) and capacity (Δ) of the reactor SBR1. Effective nitrogen loading rate applied is marked with a continuous line.

The maximum capacity of the system was calculated as the product of the maximum SAA obtained in batch tests and the concentration of biomass in the system (Figure 3.6). Along the first three periods, this capacity increased due to the growth of the biomass (from the initial 1.2 g VSS L^{-1} to 2.5 g VSS L^{-1} at the end of Period III). In Period IV, the system lost 25% of its capacity. Nevertheless, this fact did not affect the efficiency of the reactor because the effective nitrogen loading rate applied was under the maximum capacity (around 740 mg N $(L\ d)^{-1}$). Finally, in Period VI, a total loss of the system capacity took place, due to the loss in the SAA of the biomass. The obtained results agree with those reported by Jung *et al.* (2007) who found a total loss of the ammonium removal rate in the presence of FA concentrations higher than 32 mg NH_3-N L^{-1}.

Despite the descent in the average particle size (calculated by means of the volume of particles) from 1.43 mm at day 6 to 1.16 mm at day 187, good retention of the biomass was maintained due to the high relative density of the particles with inorganic carrier (Fernández *et al.*, 2008). Therefore, the presence of the commented levels of FA in the reactor did not cause problems of biomass wash-out and the concentration of biomass in the effluent remained below 10 mg VSS L^{-1}, which implied solids retention times higher than 130 d.

At the end of the experimental period, the reactor was fed again with a stoichiometric ammonium to nitrite molar ratio, to restore the system capacity. This restoration took place within 1 month (data not included).

3.4.3. Long-term effects of free nitrous acid

In a first attempt to study the long-term effects of FNA on the Anammox process, the reactor SBR2 was operated without argon flushing in its headspace. In this "Previous Period", the reactor was fed with an ammonia inlet concentration of 150 mg NH_4^+-N L^{-1}, while the nitrite inlet concentration was gradually increased from 150 to 225 mg NO_2^--N L^{-1}. Although nitrite applied was higher than that stoichiometrically required,

this compound was not accumulated in the reactor (Figure 3.7). On the other hand, nitrate concentration in the effluent was higher than that expected for the Anammox process. Therefore, the possible oxidation of nitrite into nitrate could have been occurring.

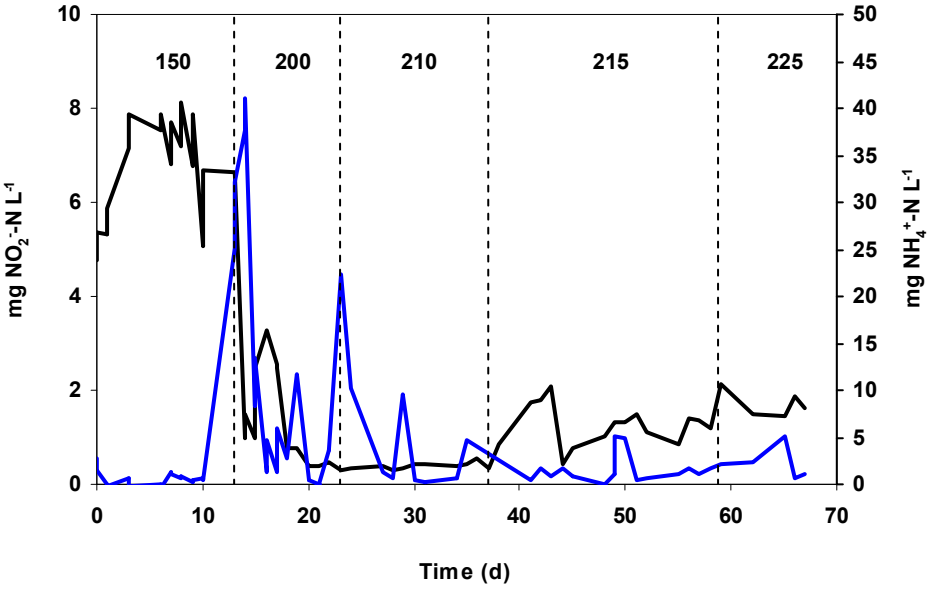

Figure 3.7. Concentrations of nitrite (—) and ammonium (—) in the effluent of SBR2 during the "Previous Period". Nitrite influent concentrations are indicated in the upper part of the figure (mg NO_2^--N L^{-1}).

To confirm the oxidation of nitrogen in the reactor, nitrifying activity tests were done to determine the possible presence of ammonia- and/or nitrite-oxidizing bacteria. The specific ammonium oxidizing activity was only 0.043 g O_2 (g VSS d)$^{-1}$, but the specific nitrite oxidizing activity was around ten times higher (0.36 g O_2 (g VSS d)$^{-1}$). Furthermore, the oxygen affinity constant K_O was calculated according to a Monod-type kinetics for the nitrite oxidizing organisms, being 0.11 mg O_2 L^{-1}. This is a value much lower than the ones reported in the literature (Hunik *et al.*, 1994; Nowak *et al.*, 1995; Guisasola *et al.*, 2005), which were within the range 0.5-1.75 mg O_2 L^{-1}. This fact might be explained if the nitrite oxidizing population

was adapted to the low oxygen conditions of the reaction media. Although the low observed K_O was not reported in other works, the detection of nitrifying activities in Anammox reactors is not uncommon, because nitrogen oxidizing populations are known to appear together with Anammox bacteria, even when the Anammox sludge is substantially enriched (van de Graaf *et al.*, 1996; Kindaichi *et al.*, 2007).

To avoid nitrite oxidation, the headspace of the reactor was flushed with argon (second attempt). This caused a strong decrease of nitrite oxidizing activity to 0.076 g NO_2^--N (g VSS d)$^{-1}$ at Period I (Table 3.1). The ammonium oxidizing activity was under the limit of quantification of the method. The nitrite oxidizing activity further decreased and, from day 218 to the end of the operation, it was also below the limit of quantification of the method. Therefore, it could be assumed that all the transformations of nitrogenous compounds were carried out by the Anammox bacteria.

The reactor was operated for 281 days. Free nitrous acid concentrations during the first two periods (Figure 3.8) were almost negligible. In Period III the system was operated in presence of 0.3 µg HNO_2-N L^{-1} and the maximum treatment capacity was not affected (Figure 3.9).

A failure of the temperature controller took place during Period IV and the inlet nitrite concentration was decreased to 200 mg NO_2^--N L^{-1} (days from 119 to 132) in order to achieve a fast restoration of the system. Once this problem was solved, inlet nitrite concentration was increased to 240 mg NO_2^--N L^{-1} (Period V) and an average FNA concentration of 0.5 µg HNO_2-N L^{-1} was achieved. No inhibitory effects took place at this FNA concentration and the capacity of the system was similar to that measured during Periods II and III.

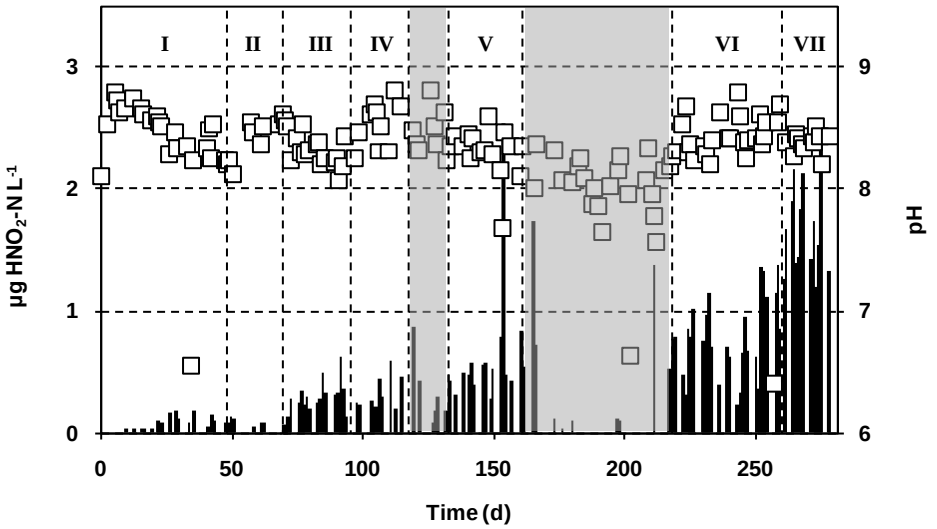

Figure 3.8. Free nitrous acid concentration (bars) and pH (□) in SBR2. Shaded areas correspond to instability and recovery periods.

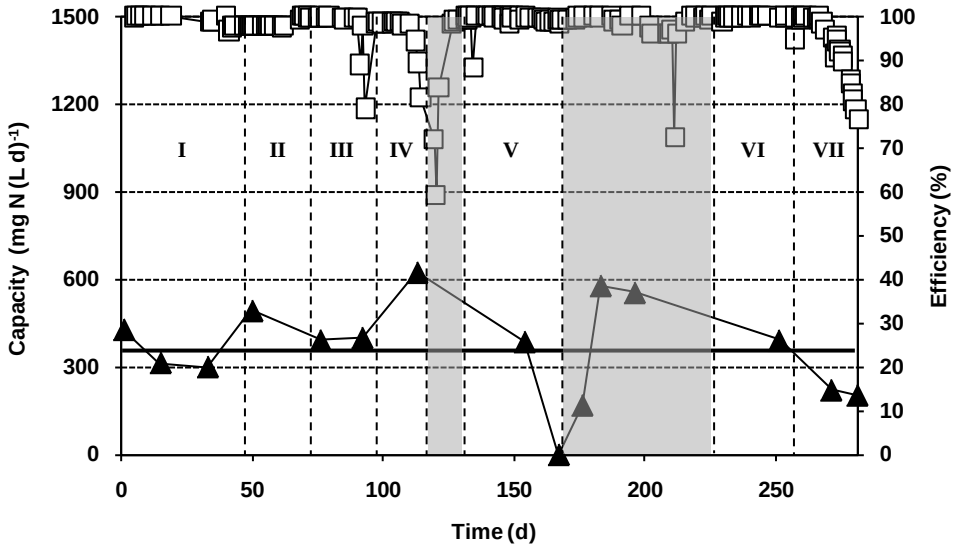

Figure 3.9. Efficiency (□) and maximum capacity (▲) of the system (SBR2). Effective nitrogen loading rate applied is marked with a continuous line. Shaded areas correspond to instability and recovery periods.

The period between days 160 and 222 corresponds to another unstable stage due to operational problems and its restoration time. After this recovery, during Periods VI and VII, the reactor was operated in the presence of average FNA concentrations of 0.7 and 1.5 μg HNO_2-N L^{-1}, respectively. For these concentrations a decrease of the capacity was observed. Furthermore, the efficiency of the treatment in terms of limiting substrate (ammonium) removal was 77% at the end of Period VII, when the maximum capacity was well below the NLR applied. Despite that loss of efficiency and the corresponding nitrite accumulation, flotation events like the ones reported by Dapena-Mora *et al.* (2004c) were not observed. Therefore the biomass retention was good with a concentration of biomass in the effluent lower than 13 mg VSS L^{-1} which implied solids retention times higher than 187 d (calculated with an average value for biomass concentration in the reactor of 2.44 g VSS L^{-1}). Furthermore, the average particle size (calculated by means of the volume of particles) increased from 1.05 mm at day 42 to 1.48 mm at day 274. The use of a high density inorganic carrier was probably the main reason for the good retention of biomass.

From day 281 the reactor was again fed with a stoichiometric ammonium to nitrite molar ratio and less than 1 month was necessary in order to restore the SAA to values similar to those measured at the beginning of the Period VI (data not shown).

The results of the present work are similar to those found by Fux *et al.* (2004). These authors reported 80% of activity loss when they operated a fixed bed reactor in the presence of 80 mg NO_2^--N L^{-1} (according to the operational conditions in their reactor, this nitrite concentration corresponded to around 4 μg HNO_2-N L^{-1}). Moreover, a more recent work by Jung *et al.* (2007) reported a significant long-term inhibition in the presence of 0.8-1.2 μg HNO_2-N L^{-1} (calculated from data reported by the authors), a range that agrees very well with the results of the present work.

3.4.4. Identification of bacterial populations

Biomass samples were taken and fixed at different times along the operational periods of both reactors. The main objective of these tests was to determine which Anammox specie or species were present along the operation of the reactor. On one hand, this fact could help to compare the present results with other works. On the other hand, it was interesting to check if there was a population shift caused by the change in the properties of the feeding. Besides, taking into account the nitrite oxidizing activity in the reactor (section 3.4.3), the presence of nitrogen oxidizers was also researched (probes not included in section 3.3.6).

However, due to the presence of the inorganic carrier (zeolite) the microscope examination of the FISH hybridized samples turned out to be difficult. The inorganic materials caused autofluorescence which prevented to observe FISH positives in some samples, especially when probes were labelled with fluorescein. Therefore, nor Anammox species, neither nitrogen oxidizers were distinguished. Thus, the results of FISH technique were similar for all of the samples taken and it was not possible to observe positives with the probes Kst1275 and Ban162. The pictures shown (Figure 3.10) correspond to samples taken at the beginning of the experience with FA (day 0, SBR1), at the end of the experience with FNA (day 281, SBR2) and at the meanwhile between the two experiences.

Positives of Pla46 specific probe for Planctomycetes were observed and Planctomycetes were found to be an important part of the bacterial population of the reactors along the operation. This can be expected since Anammox bacteria are part of the mentioned Phylum (Jetten *et al.*, 2005).

Positives of probe Amx820 were also observed. This probe is specific for Anammox species *Candidatus* Brocadia anamooxidans and *Candidatus* Kuenenia stuttgartiensis. The typical Anammox compact colony was observed, which suggested that the population was stable. The same type of Anammox colony was reported by Schmid *et al.* (2003).

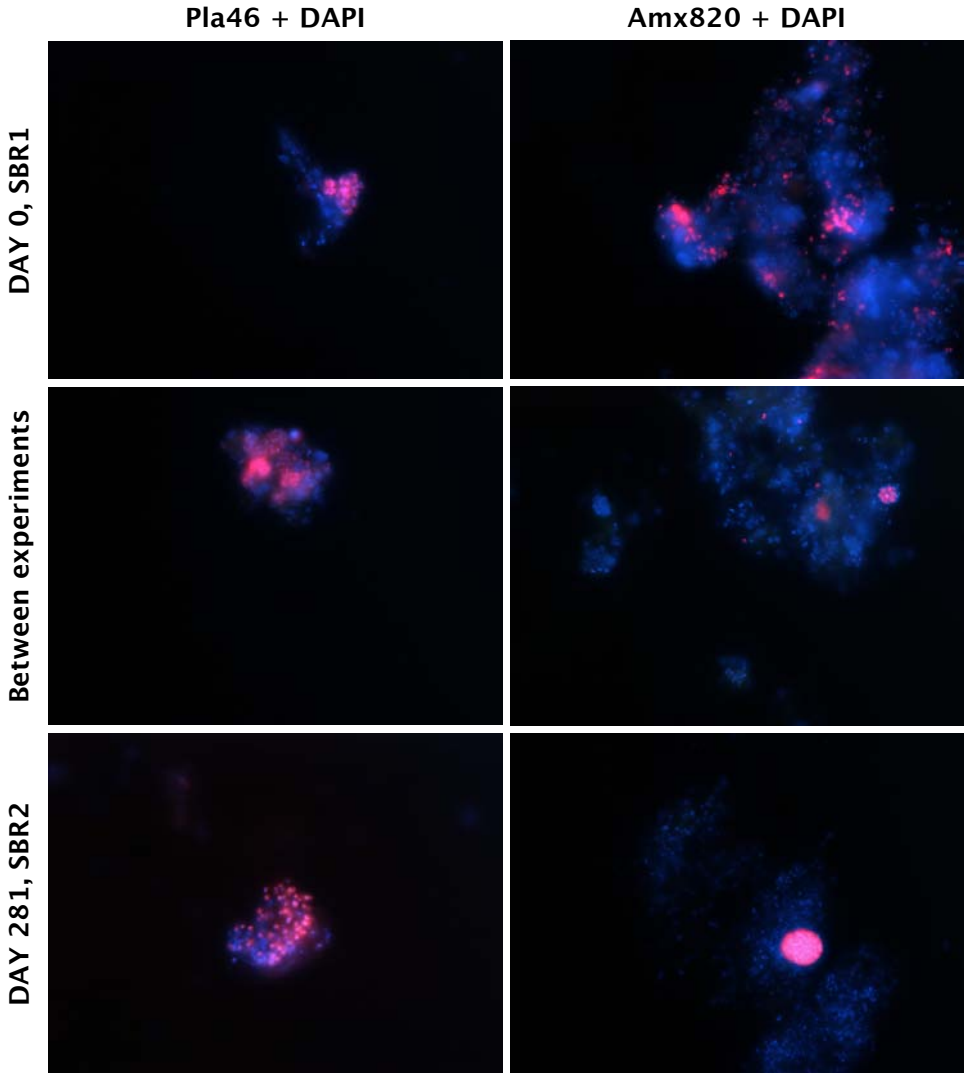

Figure 3.10. FISH micrographs (1000x). DNA is marked in blue by DAPI and Planctomycetes and Anammox are marked in pink by superposition of blue (DAPI) and red (Pla46 and Amx820, respectively).

3.5. CONCLUSIONS

Substrates were observed to have an inhibitory effect on the Anammox process in short- and long-term experiments. The effects observed were stronger at long-term and, in the case of FNA, on flocculent biomass.

Concentrations higher than 20-25 mg NH_3-N L^{-1} and 0.5 µg HNO_2-N L^{-1} should be avoided to maintain the stable operation of Anammox systems. Capacity calculations can be useful in order to predict and prevent unstable episodes.

The inhibition events caused by both substrates were reversible, the restoration time being around 1 month. The physical properties of the Anammox biofilm biomass were not substantially affected by FA or FNA.

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Chapter 4

Effects of broad-spectrum antibiotics on the stability of the Anammox process

ABSTRACT. The feasibility of the anaerobic ammonium oxidation process to treat wastewaters containing antibiotics was studied in this chapter.

The short-term effects of tetracycline hydrochloride (100-1000 mg L^{-1}) and chloramphenicol (250-1000 mg L^{-1}) on the maximum specific activity of Anammox biomass were tested in batch assays. A strong inhibitory effect was observed for both antibiotics.

A concentration of 20 mg L^{-1} of chloramphenicol was continuously added to an Anammox Sequencing Batch Reactor (SBR) system, causing a decrease of the nitrogen removal efficiency of 25%. The Specific Anammox Activity (SAA) of the biomass also decreased from 0.25 to 0.05 g N (g VSS d)$^{-1}$. Similar effects were observed when 50 mg L^{-1} of tetracycline hydrochloride were continuously fed to the reactor. Both antibiotics did not cause any changes in the physical properties of the biomass, which allowed good biomass retention.

A previous degradation step could be necessary in order to use the Anammox process to treat wastewaters containing inhibitory concentrations of antibiotics.

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[1] Some parts of this chapter have been published as: **Fernández I., Mosquera-Corral A., Campos J.L., and Méndez R.** (2009). Operation of an Anammox SBR in the presence of two broad-spectrum antibiotics. *Process Biochemistry*, **44**, 494-498.
Selected results were also presented as: **Fernández I., Mosquera-Corral A., Campos J.L. and Méndez R.** Effect of broad-spectrum antibiotics on Anammox granular biomass. International Water Conference (IWC'2006). Porto, Portugal, June 2006.

4.1. INTRODUCTION

Anaerobic digestion has being widely implemented to treat a variety of wastewater streams, from manure (Arikan *et al.*, 2006) to pharmaceutical wastewater (Ince *et al.*, 2002; Oktem *et al.*, 2007), allowing to recover energy in form of biogas. However, the effluent of the anaerobic digesters could not be ready to discharge directly into the environment due to its high ammonia concentration. In the case of these effluents, conventional nitrification–denitrification processes are, in many cases, not advisable for a post-treatment since biodegradable organic matter was previously removed by the anaerobic treatment. An alternative, in order to treat this kind of effluents, is the combination of a partial nitrification, where the 50% of the ammonium is oxidized to nitrite, and the Anammox process (Strous *et al.*, 1999). This strategy would permit the reduction of costs compared to the traditional system, because 40% less oxygen is required and the addition of organic matter is not necessary. Another advantage is the low amount of surplus sludge that would also lead to a reduction in the operational costs (Jetten *et al.*, 1997).

Due to the broad administration of antibiotics to the different kinds of livestock, the presence of these compounds is expected in the produced manure (Huang *et al.*, 2001). Several articles have been published about the fate of antibiotics in anaerobic lagoons (Loftin *et al.*, 2005; Kolz *et al.*, 2005) and anaerobic digesters (Massé *et al.*, 2000; Chelliapan *et al.*, 2006; Carballa et al., 2007; Oktem *et al.*, 2007; Arikan, 2008). Despite the inhibition by antibiotics was reported in literature, the feasibility of the anaerobic treatment of manure containing antibiotics is in general demonstrated.

The efficiency of a nitrifying unit in the presence of chloramphenicol and oxytetracycline has been previously studied by Campos *et al.* (2001). These authors reported no inhibitory effects on the nitrification with

chloramphenicol concentrations up to 250 mg L^{-1} and oxytetracycline concentrations up to 100 mg L^{-1}.

Apart from the fact that conventional processes for the removal of organic matter may potentially be affected by the presence of antibiotics, these compounds are not efficiently degraded. Specifically, Buntner *et al.* (2008) reported variable antibiotic degradation efficiencies during conventional treatments, which, in many cases led to the presence in the effluent of more than 50% of the initial concentration.

Therefore, the presence of antibiotics can be expected in some effluents from anaerobic digestion. This fact may have a negative influence if these streams are subsequently treated by the Anammox process. And, despite Molinuevo *et al.* (2009) demonstrated that livestock wastewaters can be successfully treated by the Anammox process, their work was focused on the effect of the organic matter and they did not research about the presence or effects caused by antibiotics. Actually, there are very few articles dealing with the behaviour of Anammox biomass in the presence of antibiotics and most of them were focused on short term exposure and effects. Some long term experiments were carried out by Makuch *et al.* (2007) working with three sulphonamides. These authors reported very slight effects on the performance of the process working with 80 mg L^{-1} of sulphacetamide, 40 mg L^{-1} of sulphanilamide or up to 1 g L^{-1} of p-toluene-sulphonamide.

However, it would be necessary to assess the potential effects of more types of antibiotics, especially broad-spectrum ones. Among these broad-spectrum antibiotics, two of the most employed to treat livestock are tetracyclines and chloramphenicol. In fact, Zhao *et al.* (2010) analyzed manure samples collected from several livestock feedlots and they reported very high occurrence of chlortetracycline in pig, chicken and cow manure, with concentrations up to 60 mg kg^{-1}. Arikan (2008) studied the fate of chlortetracycline during the anaerobic digestion of manure from

calves. They medicated beef calves with the usual therapeutic concentration and subsequently, they treated the obtained manure (5-fold diluted) by anaerobic digestion. They reported concentrations of chlortetracycline and its epimer and metabolite in the range of milligrams per litre after 33 days of digestion. Regarding chloramphenicol, the expected concentration in manure may be roughly 0.3 g kg^{-1} (WHO, 2005) when pigs, cattle or chicken are treated with the usual therapeutic dose. Despite the subsequent anaerobic treatment, the concentration of chloramphenicol in the effluent can be important due to its persistence (WHO, 2005).

4.2. OBJECTIVES

The first objective of this work was to evaluate the short-term effects of tetracycline hydrochloride and chloramphenicol on the Anammox activity. For this purpose, Specific Anammox Activity batch tests were employed. Furthermore, the biotoxicity of both antibiotics was assessed by standard toxicity tests.

The next objective was to evaluate the long-term effects of the mentioned antibiotics. Each antibiotic was added to the feeding of an Anammox reactor maintaining a constant nitrogen loading rate. These experiments were focused on the effects on the capacity of the treatment, its efficiency and the physical properties of the biomass.

4.3. MATERIALS AND METHODS

4.3.1. Specific Anammox activity tests

Batch experiments to determine the Specific Anammox Activity (SAA) were performed according to the methodology described in Chapter 2 (Dapena-Mora *et al.*, 2007). The tests consisted of the measurement of the overpressure generated by the produced nitrogen gas along time. They were performed at least by duplicate.

The same tests were used in order to study the short-term effects of the assayed antibiotics. The concentrations tested were 0, 250, 500 and 1000 mg L^{-1} for chloramphenicol and 0, 100, 200, 250, 500 and 1000 mg L^{-1} for tetracycline hydrochloride. The expected concentrations in the digested manure may be lower (Section 4.1) than some of the ones tested. However, the highest concentrations were employed in order to estimate the 50% inhibition concentration (IC50). When short-term effects were studied, SAA was calculated from the initial production of nitrogen, during the first 2 or 3 hours of the batch tests, when its rate of production was constant. Experiments were done at least by duplicate.

4.3.2. Experimental set-up

Experiments to determine the long-term effects of antibiotics on the Anammox process were carried out in a Sequencing Batch Reactor (SBR) of 1 L of useful volume (Figure 4.1). Dimensions of the unit were: height 150 mm and inner diameter 100 mm. The reactor was operated in cycles of 6 h distributed in four periods: 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 25%, so the Hydraulic Retention Time (HRT) was maintained at 1 day. The control of the operational cycle was performed with a PLC system Siemens model Simatic S7-200 CPU224. Two peristaltic pumps were employed to feed and draw the unit. Temperature was controlled at 31 ºC by using a thermostatic system. The pH value was not controlled and ranged between 7 and 8. The complete mixture inside the reactor was achieved using a mechanical stirrer (100 rpm).

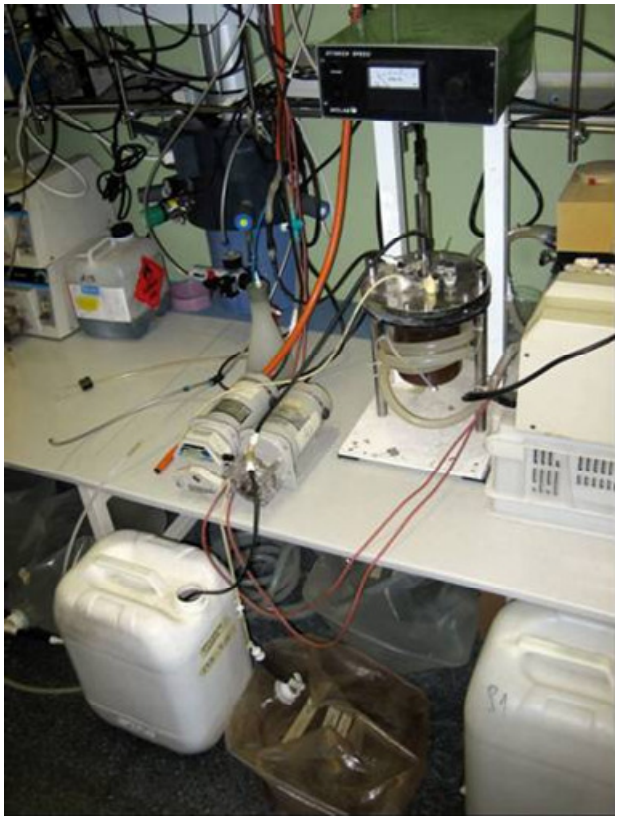

Figure 4.1. Anammox Sequencing Batch Reactor.

4.3.3. Feeding media and operational strategy

The reactor was fed with a synthetic autotrophic medium described in Section 2.4 (Dapena-Mora *et al.,* 2004a). Both ammonium and nitrite concentrations in the feeding media were 150 mg N L^{-1}, nitrite being the limiting substrate. The Effective Nitrogen Loading Rate (ENLR), calculated according to Section 3.3.3, was kept constant at 0.27 g N $(L\ d)^{-1}$ during the operation in presence of both antibiotics. In the last periods of the operation, ENLR was reduced in order to recover the treatment capacity of the reactor.

4.3.4. Biomass characteristics

The reactor was inoculated with enriched granular Anammox sludge, with a mean diameter of 1 mm, from a laboratory scale SBR (Dapena-Mora *et*

al., 2004a). This granular biomass had a Sludge Volume Index (SVI) of 43 mL g VSS^{-1}. The initial biomass concentration was 1.25 g VSS L^{-1} and its SAA was 0.25 g N (g VSS $d)^{-1}$ when chloramphenicol was assayed. When the effect of tetracycline hydrochloride was studied, the initial biomass concentration was 3.0 g VSS L^{-1} and its SAA was 0.26 g N (g VSS $d)^{-1}$. After the experiment with chloramphenicol, enriched biomass had to be added to the reactor in order to obtain sludge with the appropriate SAA for the experiment with tetracycline. This is the reason for the higher concentration of biomass during that second experiment.

4.3.5. Biotoxicity tests

Biotoxicity assays were carried out with luminescent bacteria (*Photobacterium Phosphoreum*) using the Microtox® toxicity test (ISO 11348-3: 1998). The values of concentration that caused 50% of light production inhibition (IC50) were determined after 5 and 15 min of exposure to the tested compound.

4.4. RESULTS AND DISCUSSION

4.4.1. Short-term effects

Batch tests carried out in presence of chloramphenicol and tetracycline hydrochloride showed two different profiles of gas production (Figure 4.2). The profiles obtained in presence of the former compound showed a constant gas production rate during the whole assay. The same linear trend was observed for the blank tests done without any antibiotic (data not shown). However a decrease of the gas production rate was observed in few hours when the effect of tetracycline hydrochloride was tested. Since concentrations of both substrates were high enough to avoid kinetic limitations (Dapena-Mora *et al.*, 2007), this fact would indicate a possible quick deactivation of biomass in presence of tetracycline hydrochloride.

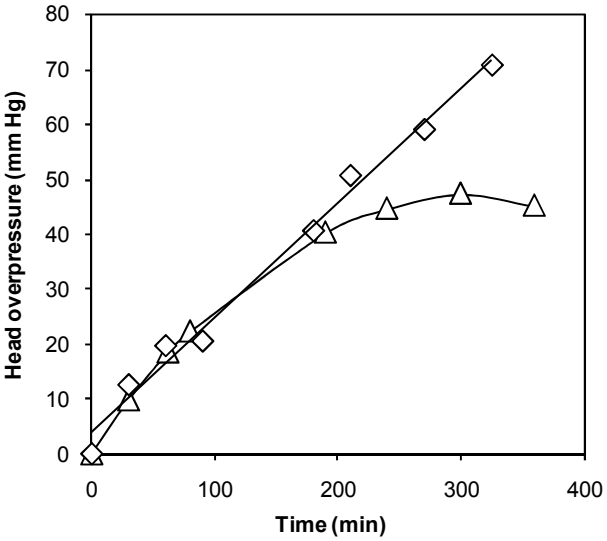

Figure 4.2. Overpressure by nitrogen production in SAA tests with 200 mg L^{-1} of tetracycline hydrochloride (Δ) and with 250 mg L^{-1} of chloramphenicol (◇).

The estimated SAA obtained at different antibiotics concentrations showed that the inhibitory effects of tetracycline hydrochloride were higher than those produced by chloramphenicol at the lower concentrations assayed (Figure 4.3). However, the effects of both compounds were similar at concentrations higher than 500 mg L^{-1}.

Results from the biotoxicity assays carried out at 5 and 15 min showed a stronger effect in the case of tetracycline hydrochloride for both exposition times (Table 4.1) compared to the ones obtained for chloramphenicol. These results agree with those obtained in the batch tests. The values of IC50 determined by both methods are very close in the case of chloramphenicol, while the value of IC50 determined by the biotoxicity assay for tetracycline hydrochloride is lower than that obtained in batch SAA tests. Furthermore, in the case of tetracycline hydrochloride, the IC50 value obtained for an exposition time of 5 min is notably higher than that corresponding to 15 min which would confirm the increase of its toxic effect with time.

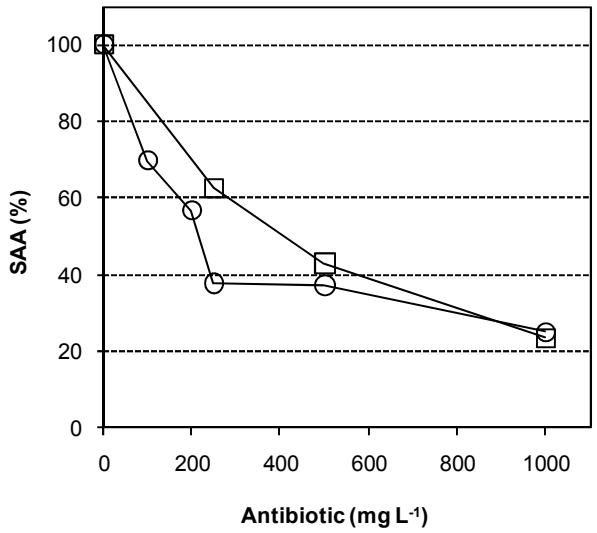

Figure 4.3. Inhibition caused by tetracycline hydrochloride (○) and chloramphenicol (□) on the SAA (average values).

Table 4.1. Biotoxicity of the antibiotics.

	IC50 (5 min) (mg toxic L^{-1})	IC50 (15 min) (mg toxic L^{-1})
Chloramphenicol	420	390
Tetracycline hydrochloride	94	42

The strong short-term inhibitory effects on Anammox biomass by tetracycline hydrochloride do not agree with the results found for nitrifying microorganisms. Gómez *et al.* (1996) studied the effects caused by five different antibiotics on the nitrification process. Among these antibiotics they used oxytetracycline, which is similar in structure and properties to tetracycline hydrochloride. They reported no significant effects observed on growth rate and nitrification rate with concentrations of the antibiotic up to 250 mg L^{-1}. On the contrary, in the case of anaerobic bacteria, Sanz *et al.* (1996) reported important inhibitory effects

by chlortetracycline. They measured a value of IC50 (15-20 mg L^{-1}) much lower than the one obtained in the present work for Anammox biomass.

Regarding the effects of chloramphenicol, van de Graaf *et al.* (1995) found that 200 mg L^{-1} of this antibiotic caused an inhibition of 68% on the Anammox activity measured in batch tests. However, they also observed that the inhibition percentage depended on the enrichment degree of the culture and the adaptation of micro-organisms. Okpokwasili and Eleke (1997) tested the effect of this compound on nitrifying biomass and observed the total inhibition of pure cultures of *Nitrosomonas* and *Nitrobacter* by chloramphenicol at concentrations of 13.3 mg L^{-1} in batch assays.

4.4.2. Long-term effects.

The Anammox SBR was started-up at a constant ENLR of 0.27 g N $(L\ d)^{-1}$ with nitrite removal efficiencies close to 100% (Figure 4.4). On day 18, a concentration of 20 mg chloramphenicol L^{-1} was added to the feeding medium. Only 4 days after chloramphenicol addition, the efficiency of the reactor began to decrease to a value of 75%. On day 38, the antibiotic was removed from the feeding but the reactor efficiency was not restored. Full efficiency was achieved again only when ENLR was reduced to 0.09 g N $(L\ d)^{-1}$ (day 58). The presence of chloramphenicol caused a decrease of activity to 20% of the initial SAA (day 38) which is related to the loss of efficiency of the reactor (Figure 4.3). After the feeding was changed back to the synthetic wastewater without antibiotic, SAA of the biomass started to recover; this would indicate a reversible effect of the inhibitory compound. However, after 2 months it was possible to recover just 59% of the initial value of SAA. Therefore the reversibility of the inhibition may be only partial.

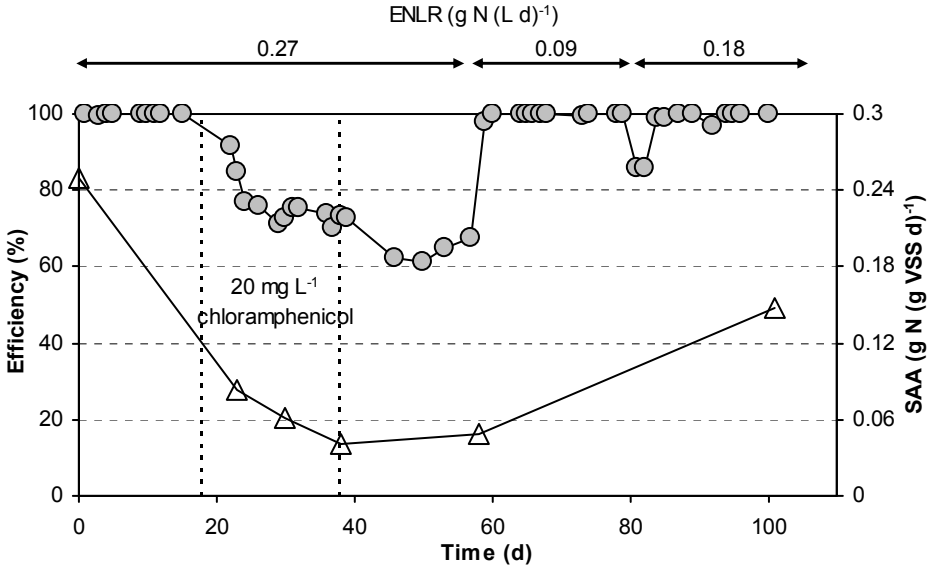

Figure 4.4. Reactor efficiency (●) and SAA of the biomass (Δ) in the experiment with chloramphenicol.

The presence of chloramphenicol in the feeding caused an increase of nitrite concentration in the reaction medium up to 40 mg N L^{-1} (data not shown). In a previous work by Dapena-Mora *et al.* (2004b) it is reported that nitrite accumulation in Anammox systems caused flotation events, which led to incidents of biomass wash-out and, consequently, to the total loss of the reactor efficiency. Nevertheless, this fact was not observed in the present study. The Anammox granules showed no change on their appearance when observed with the stereomicroscope (Figure 4.5).

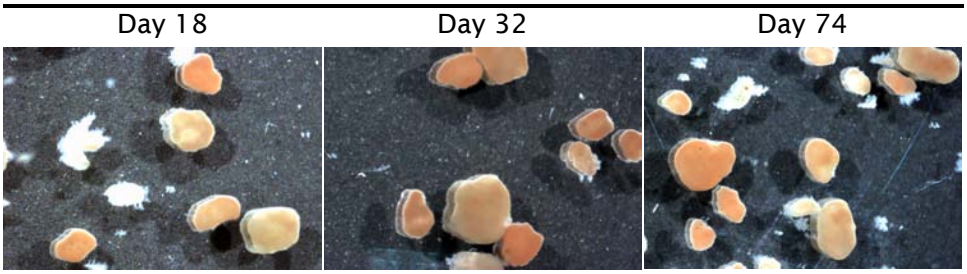

Figure 4.5. Stereomicroscope observation of Anammox granules (10X) taken from the reactor during the experience with chloramphenicol.

Furthermore, neither the size distribution of the granules (Figure 4.6) nor the physical properties of the Anammox granules were affected by the antibiotic or the nitrite accumulation. These parameters were practically constant at values of 42.9 mL g VSS^{-1}, 104 g VSS $(L_{granules})^{-1}$ and 1.0 mm, corresponding to the SVI, biomass density and mean diameter, respectively. The good settleability of biomass allowed maintaining concentrations of volatile suspended solids in the effluent between 3 and 5 mg VSS L^{-1}. Therefore, the solids retention time was higher than 400 days.

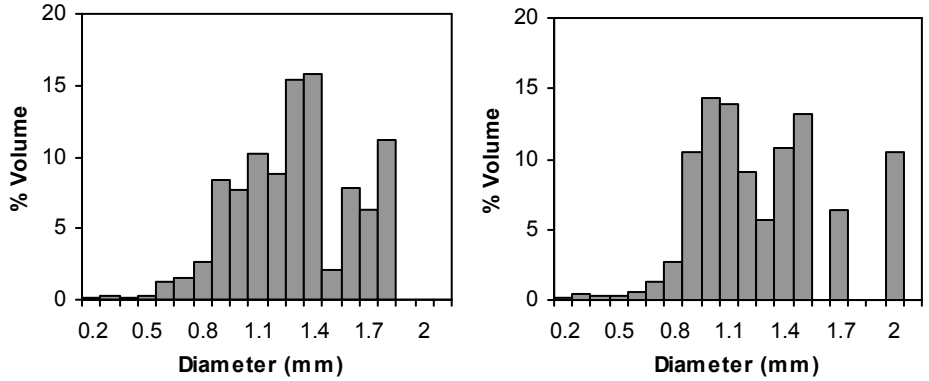

Figure 4.6. Distribution of particle size in percentage of volume at the beginning (left) and the end (right) of the experiment with chloramphenicol.

To assay the long-term effect of tetracycline hydrochloride, the concentration of this compound applied to the Anammox reactor was stepwise increased from 0 to 50 mg L^{-1}. An antibiotic concentration of 10 mg L^{-1} decreased the SAA of biomass to around 40% of the initial value (Figure 4.7) but due to the fact that the system was not operated at its maximum capacity, no nitrite accumulation occurred as it was stated in previous works (Dapena-Mora *et al.*, 2004b). When the inlet tetracycline hydrochloride concentration was increased to 50 mg L^{-1}, the SAA decreased to 37% of its initial value. Although this decrease was only slightly higher than that observed in the presence of 10 mg L^{-1} of antibiotic it was enough to produce nitrite accumulation in the system.

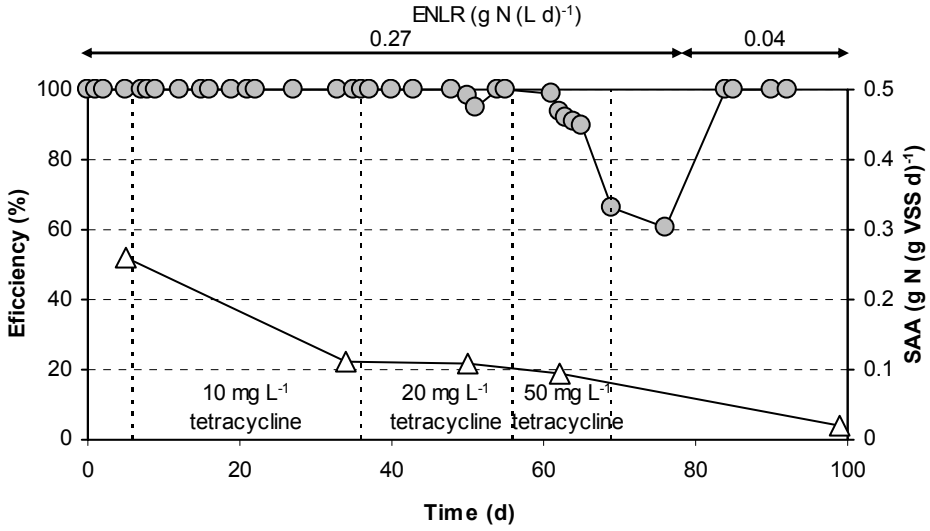

Figure 4.7. Reactor efficiency (●) and SAA of the biomass (Δ) in the experiment with tetracycline hydrochloride.

From day 70, the antibiotic was not fed to the reactor and the ENLR was reduced to 0.04 g N $(L\ d)^{-1}$ in order to recover the biomass activity. This recovery was not possible after more than 1 month of operation without the tetracycline; on the contrary, SAA continued decreasing and it was almost negligible at the end of the experiment. This irreversible loss of

activity may be probably related to the mentioned deactivation capability of tetracycline hydrochloride (Section 4.4.3).

As in the case of chloramphenicol, tetracycline hydrochloride did not cause any effect over the physical properties of the granules, remaining their aspect unchanged (Figure 4.8).

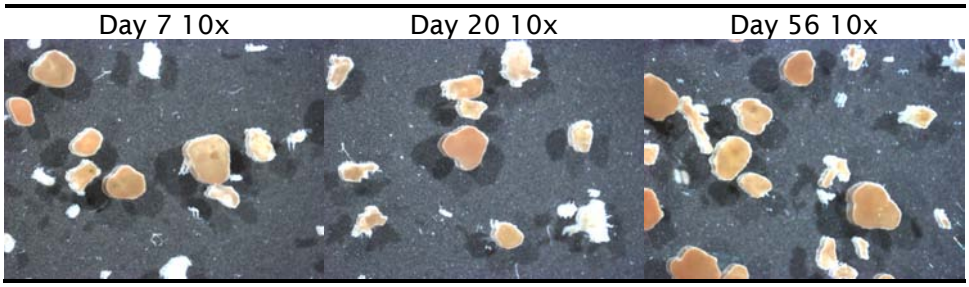

Figure 4.8. Stereomicroscope observation of Anammox granules taken from the reactor in the experiment with tetracycline hydrochloride.

Furthermore, the values of the SVI and the density of the biomass were almost constant along the experiment. Their values were 42 mL g VSS^{-1} and 81 g VSS L^{-1} and the average particle size (Figure 4.9) was around 1.1 mm during the whole operational period. The granular condition of the biomass was maintained according to the criteria established by de Kreuk *et al.* (2007). The good settling properties led to a very low biomass concentration in the effluent, always under 12 mg VSS L^{-1} and, consequently, the system operated at SRT higher than 250 days.

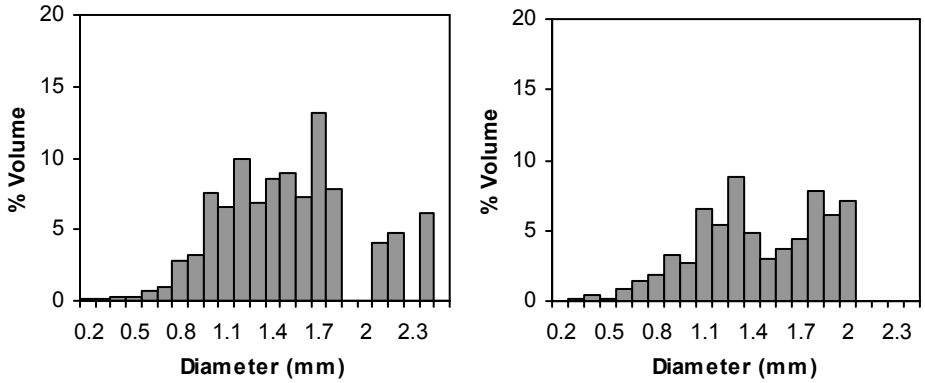

Figure 4.9. Distribution of particle size in percentage of volume at the beginning (left) and the end (right) of the experiment with tetracycline hydrochloride.

In literature, there are no references about the effect of tetracycline on the Anammox process. For nitrifying bacteria 50% of inhibition at 250 mg L^{-1} of oxytetracycline, which is similar in structure and properties to tetracycline hydrochloride, was reported in the continuous operation (Campos *et al.*, 2001). Recently, Prado *et al.* (2009) were researching its effects on the biological treatment of swine wastewater employing a semi-industrial membrane bioreactor with an anaerobic zone. However, in their case the concentrations measured on the different points of the reaction system were lower than the tested ones on the present work (5 mg L^{-1} as maximum). Therefore, the authors reported no effect on the elimination of organic matter or on the nitrification.

According to the present work, in order to apply the Anammox process to remove nitrogen from wastewaters with potentially inhibitory concentrations of antibiotics, some specific degradation techniques should be applied to avoid the negative effects. In this sense, heterogeneous photo-degradation or chlorination could be used as proposed by Addamo *et al.* (2005) and Qiang *et al.* (2006), respectively. More work with effluents of both, pharmaceutical industry and anaerobic

digesters treating pig slurry should be carried out in order to complete the present results.

4.5. CONCLUSIONS

A strong inhibition by both antibiotics of the SAA was observed in both, batch and continuous tests. Batch tests showed that tetracycline hydrochloride exerted a deactivation effect on the biomass. During continuous tests, the addition of both compounds caused a decrease on the efficiency of the system related to a decrease of the SAA. A previous degradation step is advisable in order to treat wastewaters with antibiotics by the Anammox process.

The antibiotics tested did not cause any change in the physical properties of the biomass, so the biomass retention capacity was not affected even during unstable periods.

The inhibition by chloramphenicol was reversible, so the system could recover its efficiency without a reinoculation. The tetracycline hydrochloride caused deactivation of the biomass, so long-term exposures could lead to the need of a reinoculation of the reactor.

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Chapter 5

Start up and operation of Anammox reactors at moderately low temperatures

ABSTRACT.- The application of the Anammox process has been usually focused on the treatment of wastewater with temperatures around 30 ºC in order to operate under optimum conditions. In this chapter, the feasibility of the application of the Anammox process at lower temperatures has been tested.

Firstly, the short-term effects of temperature on the Anammox biomass were studied using batch tests. An activation energy of 63 kJ mol^{-1} was calculated and the maximum activity was found at 35-40 ºC.

In a second step, a Sequencing Batch Reactor (SBR) was operated with synthetic wastewater at gradually decreasing temperatures to determine the long-term effects. The system was successfully operated at 18 ºC but when temperature was 15 ºC, nitrite started to accumulate and the system lost its stability. Adaptation of biomass to low temperatures was observed.

Finally, the supernatant of an anaerobic digester was treated at 20 ºC in a system with a two units configuration, conformed by two SBRs, carrying out the partial nitrification and the Anammox process. Partial nitrification was achieved by granular biomass with a mean diameter of 3 mm, operating at a dissolved oxygen concentration of 2.7 mg L^{-1}. The combined system removed about 0.08 g N $(L\ d)^{-1}$.

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5.1. INTRODUCTION

Temperature is a very important factor influencing the operation of all biological wastewater treatment processes. Several authors (Strous *et al.*, 1999; Egli *et al.*, 2001; Toh *et al.*, 2002; Yang *et al.*, 2006) found that optimum temperature for the operation of the Anammox process was around 30–40 ºC. In all these works the Anammox bacteria involved belonged to the species which can be found in wastewater treatment plants or fresh water. Taking into account this optimum, the application of the Anammox process to industrial wastewater was basically focused on the treatment of effluents from anaerobic digesters operated at mesophilic conditions (Strous *et al.*, 1997; Strous *et al.*, 1998; van Dongen *et al.*, 2001; Imajo *et al.*, 2004; Caffaz *et al.*, 2006; Abma *et al.*, 2007). However, when the effluent to be treated by the Anammox process does not come from this kind of anaerobic treatment (i.e. the effluent comes at relatively low temperature) the hypothetical necessity of heating would make the treatment unacceptable in terms of energy consumption.

However, recently, Cema *et al.* (2007) proved that a rotating biological contactor (RBC) with the established Anammox process could be successfully operated at temperatures around 20 ºC. Similar results were reported by Isaka *et al.* (2007) who operated a fixed bed Anammox reactor which treated 8.1 g N $(L\ d)^{-1}$ at 20-22 ºC. Isaka *et al.* (2008) also worked with Anammox bacteria entrapped into a gel carrier and they reported nitrogen conversion rates of 2.8 and 0.36 g N $(L\ day)^{-1}$ at 22 and 6.3 ºC, respectively.

Moreover, several works done with marine Anammox samples reported measurable activities at very low temperatures (slightly below 0 ºC). Rysgaard *et al.* (2004), working with permanently cold sediments (-1.7 to 4 ºC) of the east and west coasts of Greenland, observed Anammox activity between -2 and 30 ºC, the optimum temperature being 12 ºC. Similar results were found by Dalsgaard and Thamdrup (2002) working

with marine sediments from the Skagerrak (Baltic-North Sea transition). These authors reported an optimal ammonium removal rate at about 15 ºC. Dalsgaard *et al.* (2002) reported Anammox activity from bacteria present in marine sediments between 6 and 43 ºC.

All these results indicate that the application of the Anammox process could not be restricted to effluents with temperatures around 30 ºC. Therefore, since the Specific Anammox Activity (SAA) has been demonstrated as a very useful tool in order to evaluate the behaviour of Anammox biomass at short-term experiments under different conditions (Dapena-Mora *et al.*, 2007), these tests will be appropriate to perform a first approach to the behaviour of the Anammox process at relatively low temperatures. They can also be useful for monitoring the operation of Anammox reactor at low temperatures,

5.2. OBJECTIVES

The first objective of this work was to evaluate the short-term effects of temperature on the Anammox activity. For this purpose, Specific Anammox Activity batch tests were employed.

The next objective was to evaluate the long-term effects of relatively low temperature on the Anammox process, especially the performance of the treatment and the potential acclimation of the biomass. Two different Anammox SBRs were operated and monitored in order to accomplish this objective.

5.3. MATERIALS AND METHODS

5.3.1. Specific Anammox activity tests

Batch specific activity tests were performed, according to the methodology described in Chapter 2 (Dapena-Mora *et al.*, 2007), in order

to determine the short-term effects and monitor the operation of the reactors. The tests to assess the short-term effects were done by triplicate and the range of temperatures employed was 10-45 ºC.

The maximum capacity of the Anammox systems was calculated as the product of biomass concentration inside the reactor and the maximum SAA measured in batch tests done at the operating temperature. The comparison of this maximum capacity and the Effective Nitrogen Loading Rate (ENLR; calculated according to Section 3.3.3) was used to assess the nitrogen underload or overload of the reactors.

5.3.2. Experimental set-up

Two experimental set-ups were employed in order to complete the continuous experiences.

5.3.2.1. ASBR1

To study the possibility of the acclimation of biomass to low temperatures, a lab-scale Sequencing Batch Reactor of 1 L was employed (ASBR1). The operating temperature was maintained by means of a temperature controller (PolyScience, USA). The value of pH was not controlled and ranged between 7 and 8. The control of the pumps and the stirrer, according to the different periods of the operational cycle, was performed with a PLC system (S7-CPU224, Siemens). The reactor was operated in cycles of 6 hours distributed in four periods: 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 25%, giving a Hydraulic Retention Time (HRT) of 1 day.

5.3.2.2. Partial nitrification-Anammox system

Partial nitrification was performed in a SBR with a volume of 1.5 L (NSBR; Vázquez-Padín, 2009). Its height to the diameter ratio was 5.5 and the exchange volume was fixed at 50%. The hydraulic retention time was 0.25 d. Air was supplied to the bottom of the reactor by using an air

pump. The concentration of dissolved oxygen in the liquid phase was regulated by changing the ratio of fresh air to recycled air (taken from the top of the system) injected in the reactor.

An Anammox SBR with an effective volume of 1 L (ASBR2) was used to treat the effluent from NSBR. ASBR2 was provided with a thermostatic jacket to maintain the temperature at 20 ± 1 ºC. The pH value was not controlled and remained around 7.5. Complete mixture inside the reactor was achieved with a mechanical stirrer at 100 rpm. Norprene tubing and connections were used to prevent the diffusion of oxygen into the system. ASBR2 was operated in cycles of 6 h with the same time distribution as employed for ASBR1. It was controlled with a programmable logic controller system (S7-CPU224, Siemens). The exchange volume was of 25% and the HRT was fixed at 1 d. The effluent from NSBR was collected and stored in a cold room (4 ºC) prior to feed ASBR2.

5.3.3. Feeding media and operational strategy

5.3.3.1. ASBR1

ASBR1 was fed with a synthetic autotrophic medium described in Section 2.4 (Dapena-Mora *et al*., 2004b). The ammonium to nitrite molar ratio in the feeding media was fixed at 1 (150 mg N L^{-1} of each one) to operate in excess of ammonia (Table 5.1).

The ENLR was kept constant at 0.27 g N $(L\ d)^{-1}$ and the temperature of the reactor was gradually decreased from 30 to 15 ºC during the operation (Table 5.2).

However, when the stability of the reactor failed (Period VI) the ENLR was decreased to 0.04 g N $(L\ d)^{-1}$ in order to restore the efficiency of the process.

Table 5.1. Feeding composition for ASBR1.

Feeding solution	
Compound	Concentration (mg L^{-1})
NH_4^+-N	150
NO_2^--N	150
$KHCO_3$	1.25
$CaCl_2$	1.41[a]
KH_2PO_4	50
$MgSO_4$	58.6
$FeSO_4 \cdot 7H_2O$	9.08
EDTA	6.25
Trace solution[b]	1.25 mL L^{-1}

[a] Reduced to 0.07 from Period III. [b] Described in Chapter 2.

Table 5.2. Operational Periods of ASBR1.

Period	Temperature (ºC)	Duration (d)
I	30	1- 15
II	26	15- 29
III	23	29- 49
IV	20	49- 63
V	18	63-103
VI	15	103-150

5.3.3.2. Partial nitrification-Anammox system

NSBR 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 composition of the supernatant was: pH 7.5–8.3; NH_4^+ 400–700 mg N L^{-1}; total inorganic carbon 300–505 mg C L^{-1} and total organic carbon 20–50 mg C L^{-1}. The supernatant was diluted in a proportion 1:1 with tap water prior to feed the reactor.

The strategy of operation followed with ASBR2 was similar to the one employed for ASBR1. The temperature of this Anammox reactor was

initially 30 ºC and it was stepwise decreased to 20 ºC to allow the acclimatization of the biomass. During this phase ASBR2 was fed with a synthetic medium with the following concentrations of nitrogen compounds: NH_4^+: 130-140 mg N L^{-1}; NO_2^-: 160-170 mg N L^{-1}. The concentrations of salts and trace compounds were the same as employed for ASBR1 (Table 5.1). The nitrite to ammonium ratio on molar basis of this synthetic wastewater was about 1.3, being approximately the stoichiometric one (Strous *et al.*, 1999). Once the temperature of the reactor was decreased to 20 ºC and the operation stabilized, the effluent of the granular NSBR was fed to ASBR2. The NO_2^- to NH_4^+ molar ratio was near to 1.0 and the concentration of both nitrogen compounds was about 140 mg N L^{-1}.

5.3.4. Biomass characteristics

The short-term effects of temperature were studied using both biofilm (Fernández *et al.*, 2006) and granular biomass (Dapena-Mora *et al.*, 2004c) taken from bioreactors operated at around 30 ºC ("non-adapted" biomass). Zeolite (ZeoCat, Spain) was used as the biofilm support. The particle size was between 0.5 and 1.0 mm. All the employed biomass (both, biofilm and granular) was enriched in bacteria belonging to the specie *Kuenenia stuttgartiensis*.

The ASBR1 was inoculated with 7.6 g VSS L^{-1} of the biofilm biomass (Figure 5.1) while ASBR2 was inoculated with 1.5 g VSS L^{-1} of the granular Anammox biomass, with an average diameter of 1 mm.

The nitrifying granular biomass operated in NSBR was obtained from heterotrophic aerobic granules by the stepwise decrease of the COD/N ratio in the influent (Mosquera-Corral *et al.*, 2005; Vázquez-Padín, 2009). Its concentration was 5 g VSS L^{-1}, with an average diameter of the granules around 2.8 mm.

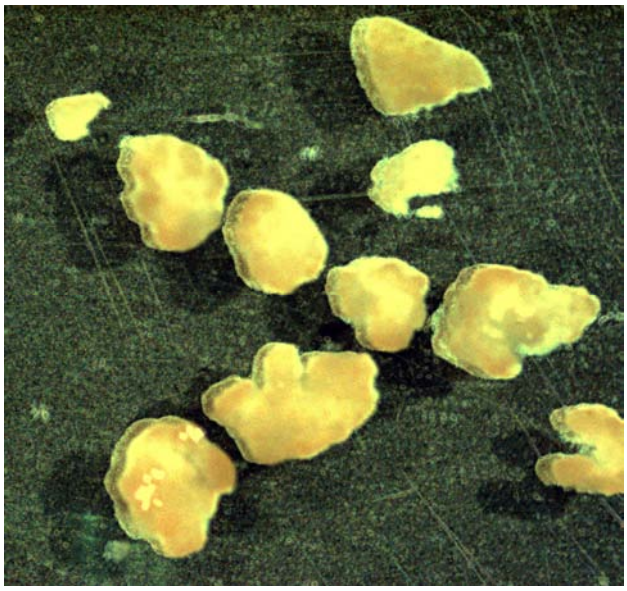

Figure 5.1. Stereomicroscope image of the inoculum of ASBR1 at the beginning of the operation.

5.3.5. UV-visible absorption spectrometry

Since liquid phase acquired an orange coloration during some of SAA tests (Section 5.4.1), the absorption spectra of those media (at the end of the experiments) were measured with a Shimadzu UV-1603 UV-visible spectrophotometer connected to a PC, to try to identify the dying compound. Hellma quartz glass cells with 1 cm of light path were used.

5.3.6. FISH

Microbial populations were monitored by the Fluorescence In Situ Hybridization (FISH) technique. Biomass samples were collected, disrupted and fixed, according to the procedure described by Amann *et al.* (1995), with 4% paraformaldehyde solution. Hybridization was performed at 46 ºC for 90 minutes adjusting formamide concentrations at the percentages shown in Table 5.3. The used probes for in situ hybridization were labelled with the fluorochromes FITC and Cy3. Fluorescence signals of disrupted samples were recorded with an acquisition system coupled with an Axioskop 2 epifluorescence microscope (Zeiss).

Table 5.3. FISH probes.

Probe	Sequence (5' → 3')	% Formamide	Reference
EUB338-I	GTC GCC TCC CGT AGG AGT	35	Amann *et al.*, 1990
EUB338-II	GCA GCC ACC CGT AGG TGT	60	Daims *et al.*, 1999
Pla46	GAC TTG CAT GCC TAA TCC	20	Neef *et al.*, 1998
Amx820	AAA ACC CCT CTA CTT AGT GCC C	35	Schmid *et al.*, 2001

5.4. RESULTS AND DISCUSSION

5.4.1. Short-term effects

The SAA of both granular and biofilm Anammox biomass were determined at temperatures between 10 and 45 ºC. The two temperature dependency profiles obtained (Figure 5.2) were very similar and consistent with the results previously reported by Strous (2000) and Egli *et al.* (2001).

An exponential increase of the SAA was observed for temperatures up to 40 ºC, while assays carried out at 45 ºC showed a negative effect of the temperature on the activity. An activation energy of 63 kJ mol^{-1} was calculated for both Anammox populations according to the Arrhenius model (Hao *et al.*, 2002). Strous *et al.* (1999) obtained a similar value (70 kJ mol^{-1}) for Anammox biomass cultivated at 30 ºC, while Dalsgaard and Thamdrup (2002) and Rysgaard *et al.* (2004) reported values of 61 and 51 kJ mol^{-1}, respectively, for Anammox biomass from marine sediments.

When assays were performed at 45 ºC the liquid phase in SAA vials acquired an orange coloration, which could indicate the biomass lysis. In order to confirm this fact, a second feeding of substrates was added inside the vials, after first feeding was consumed, and very low activity was observed (Figure 5.2).

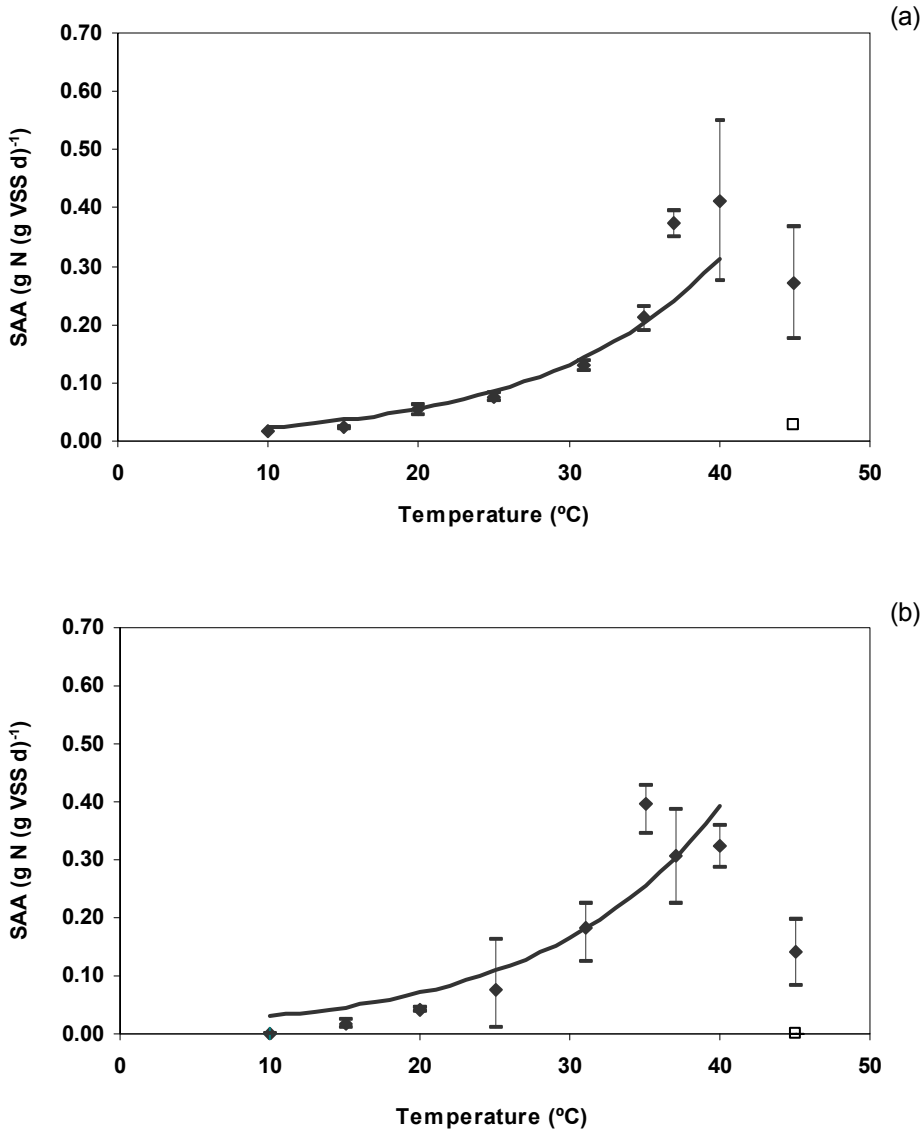

Figure 5.2. Temperature dependency profiles for granular (a) and biofilm (b) Anammox biomass (experimental SAA (♦); experimental SAA with the second injection of substrate (□); Arrhenius model (—)).

UV-visible absorption spectra of the liquid phase from the SAA batch tests done at 35, 40 and 45 ºC were analyzed (Figure 5.3). For the test done at 45 ºC, a maximum peak between 400 and 410 nm and a smaller one

between 515 and 550 nm were observed. Cirpus *et al.* (2005) analyzed the UV–visible spectrum of a 10 kDa cytochrome c present in cell extracts from a culture of *K. stuttgartiensis*. They observed a maximum absorption at 410 nm for the oxidized form of the protein. Huston *et al.* (2007) also observed a maximum peak around 410 nm and two smaller peaks at 520 and 550 nm. Therefore, the orange colour of the liquid phase at the end of the assay at 45 ºC could be attributed to biomass lysis with segregation of cytochrome c, which causes an irreversible loss of the activity.

The negative effect of high temperature was also found by Toh *et al.* (2002), which tried to select and enrich an Anammox consortium from sludge of a municipal treatment plant at 37 and 55 ºC. These authors obtained Anammox activity at a mesophilic range but they could not select thermophilic Anammox organisms at 55 ºC.

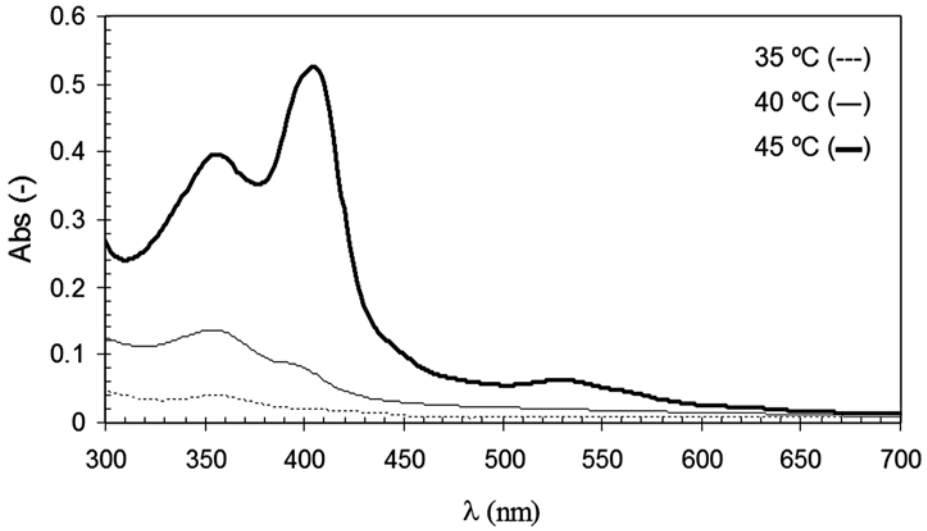

Figure 5.3. Absorbance profiles of the supernatant of the SAA tests at 35, 40 and 45 ºC.

5.4.2. Long-term effects

The biomass was progressively adapted to lower operating temperatures since a drastic change in the operational conditions could lead to a destabilization of the biological system (Szatkowska *et al.*, 2006). Figure 5.4a shows the NH_4^+-N and NO_2^--N concentrations in the effluent during the operational period. Nitrite (the limiting substrate) was totally depleted until the operating temperature was decreased from 18 to 15 ºC.

At 15 ºC the system was not able to remove all the nitrite applied and, therefore, this compound was accumulated in the reactor. As nitrite is inhibitory for the Anammox process, even at moderate concentrations (Fux *et al.*, 2004; Jung *et al.*, 2007), this accumulation caused a decrease of the capacity of the system, and then a higher nitrite accumulation, which provoked a snowball effect, causing the total loss of the system efficiency. The complete efficiency of the system was only restored when the ENLR was decreased to 0.04 g N $(L\ d)^{-1}$. However, the SAA was not recovered even after 1 month of operation at this low ENLR (data not shown). Then, the operating temperature was increased to 30 ºC as a strategy in order to increase the growth rate and restore the capacity of the system. However, this strategy was not able to improve the mentioned capacity after two and a half months of additional operation. At the end of the experiment, 3.5 months after the nitrite accumulation event, the SAA remained below 0.02 g N $(g\ VSS\ d)^{-1}$. This fact would indicate an irreversible loss of the activity that could be caused by the mixed effect of the low temperature and the presence of nitrite (Dapena-Mora *et al.*, 2004a; Strous *et al.*, 1999).

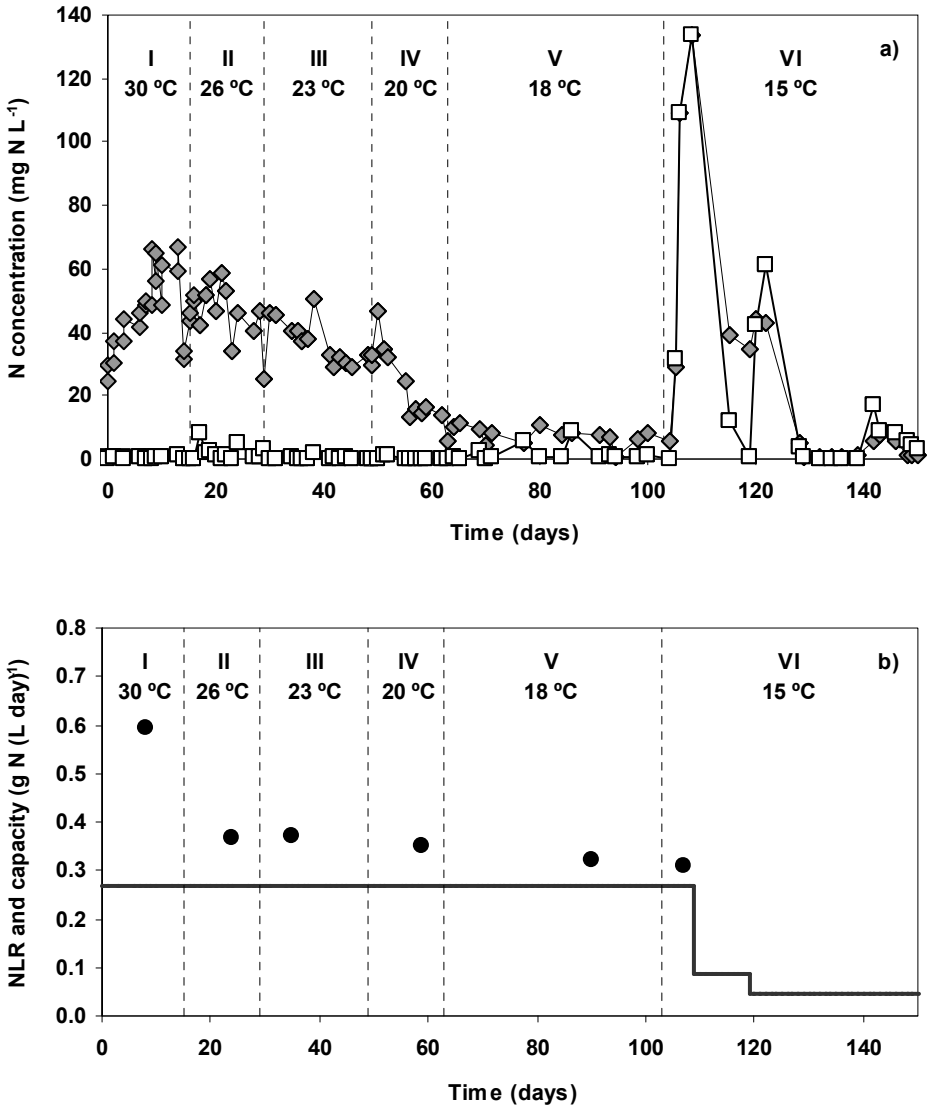

Figure 5.4. (a) NH_4^+-N (♦) and NO_2^--N (□) concentrations in the effluent and (b) ENLR (—) and maximum nitrogen removal capacity (●).

In spite of the occurrence of unstable periods, the system maintained its good biomass retention capacity and flotation events like the reported by Dapena-Mora *et al.* (2004a) were not observed. Therefore, the solids retention time, calculated as the ratio between total biomass inside the

reactor (g VSS) and the biomass wash-out rate in the effluent (g VSS d^{-1}), remained around 150 days along the whole experiment.

As it can be seen in Figure 5.4b, the maximum capacity of the reactor was decreasing along the operation, although that decrease was relatively small in the steps from 26 to 18 ºC. Taking into account that the biomass concentration was almost constant, the loss of treatment capacity observed was directly related to the decrease of the SAA.

The nitrogen removal rate observed at 20 ºC was much lower than that reported by Isaka *et al.* (2007), which could be probably explained by the high biomass concentration of their system (20 g SS L^{-1}). Cema *et al.* (2007) operated a RBC reactor at 17 ºC and obtained an average inorganic nitrogen removal rate of 0.5 g N $(L\ d)^{-1}$ which is in the range of that obtained in the present work at 18 ºC.

Figure 5.5 shows the SAA values obtained with no-acclimated biofilm biomass at different temperatures (Section 5.4.1) and those values determined during the operation of the reactor. Not only the values of SAA for adapted biomass are higher, but also the diminishing tendency is less important than in the case of non-adapted biomass. Therefore, the slow adaptation of the sludge seems to be a key factor in order to operate an Anammox reactor at low temperatures. Taking into account the very slow growth rate of the Anammox biomass (Strous *et al.*, 1999), an advisable start up strategy to operate a system at low temperatures would have two steps. The first one would be the production of the required amount of biomass, working in a separate reactor at a temperature close to the optimum. Then, the second step would be the slow adaptation of the biomass to low temperatures in the same reactor and, finally, the inoculation of the low-temperature reactor could be carried out.

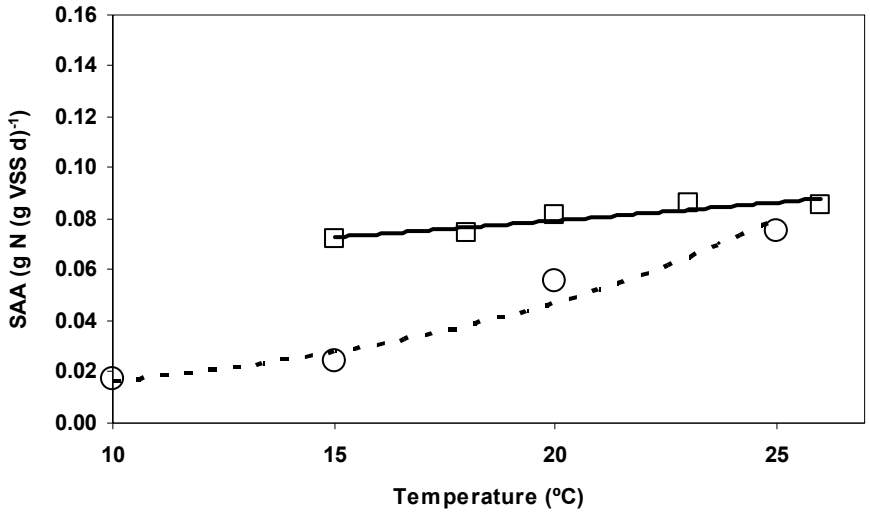

Figure 5.5. SAA of non-adapted (○) and adapted (□) Anammox biofilm biomass.

There was a change observed in the measured substrate (NO_2^--N to NH_4^+-N) consumption ratio from values close to 1.3 during the first three periods to below 1.1 when the reactor was operated at 18 ºC (Period V). Therefore the ammonium concentration in the effluent decreased during the last operational periods (Figure 5.4a). It could be attributed to two hypothetical and concurrent reasons: a) It was reported by Dalsgaard and Thamdrup (2002) that the substrate consumption ratio for Anammox biomass coming from marine sediments incubated at 15 ºC was 1. Therefore it could be a change of the Anammox substrate consumption ratio. b) Ammonium oxidation could occur (at some extent) due to the possible presence of ammonia-oxidizers which are commonly reported to appear with Anammox bacteria (van de Graaf *et al.*, 1996; Kindaichi *et al.*, 2007).

The physical characteristics of Anammox biomass were not affected by the relatively low temperatures and remained almost constant. The sludge

volumetric index was 58 mL g VSS^{-1} and the mean diameter of the granules was about 1.35 mm.

Due to the presence of the inorganic carrier (zeolite), FISH images presented autofluorescence and blurry areas. These causes made difficult the observation of positives in some cases, especially when working with probes labelled with fluorescein (FITC). Nevertheless, there were observed positives of the probes Pla46 and Amx820 (Figure 5.6) at the beginning (not shown in the figure) and at the end of the experimentation with ASBR1. The first is specific for Planctomycetes, the Phylum of Anammox bacteria (Jetten *et al*., 2005), and the second is specific for Anammox species *Candidatus* Brocadia anammoxidans and *Candidatus* Kuenenia stuttgartiensis.

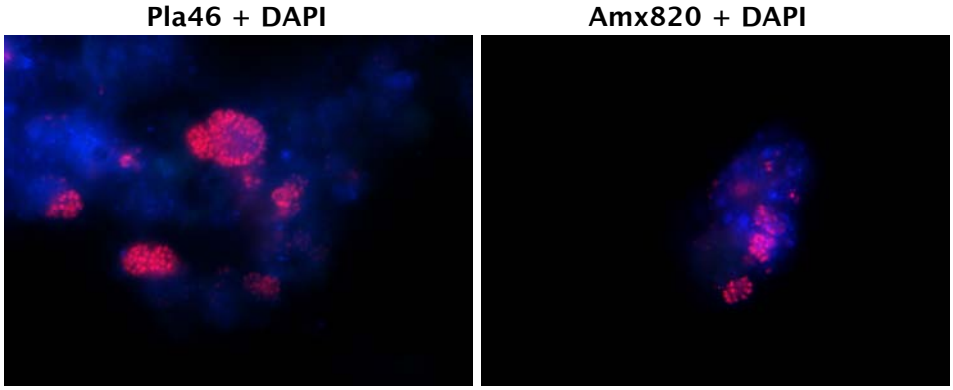

Figure 5.6. FISH micrographs (1000x). DNA is marked in blue by DAPI and Planctomycetes and Anammox are marked in pink by superposition of blue (DAPI) and red (Pla46 and Amx820, respectively).

5.4.3. Application of partial nitrification and Anammox processes

Stable partial nitrification was reached in the NSBR during 60 days (Vázquez-Padín, 2009). The obtained NO_2^-/NH_4^+ molar ratio was 1.1 ± 0.2; the concentrations of both ammonium and nitrite in the effluent were around 140 mg N L^{-1}.

During the first acclimation period the Anammox reactor ASBR2 was fed with a synthetic media (Section 5.3.2.2; Figure 5.7) and the temperature was stepwise decreased from 30 to 20 ºC, reaching the latter on day 20. The estimated SAA decreased from 0.28 to 0.13 g N (g VSS d)$^{-1}$ due to the temperature decrease. On day 33 of operation of the Anammox reactor, the synthetic wastewater was switched to the effluent of the granular SBR. After that, the mean efficiency of the Anammox reactor in terms of nitrogen removal decreased from 80% to 69% due to the operation under nitrite limitation. The global nitrogen removal rate of the two units system was about 0.08 g N (L d)$^{-1}$.

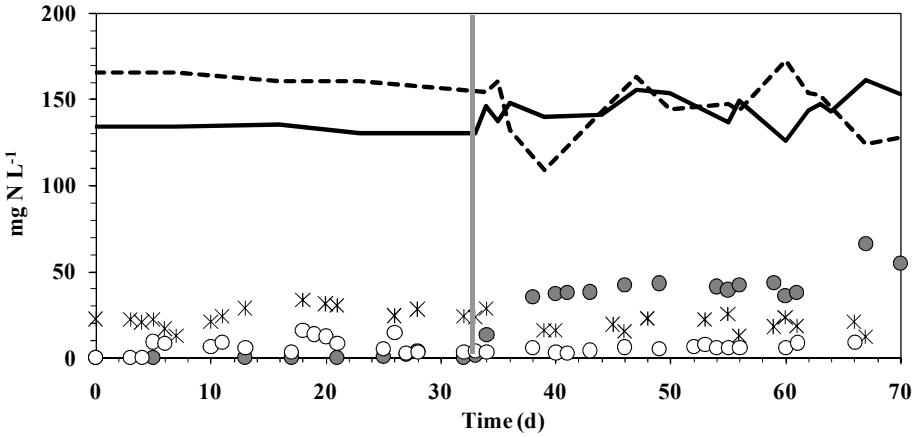

Figure 5.7. Concentrations of nitrogen compounds in the influent: mg NH_4^+–N L^{-1} (—) and mg NO_2^-–N L^{-1} (- - -); and in the effluent mg NH_4^+–N L^{-1} (●), mg NO_2^-–N L^{-1} (○) and mg NO_3^-–N L^{-1} (*).

Biomass concentration inside the Anammox SBR did not change significantly and it was around 1.5 g VSS L^{-1} during the whole operational period. Solids concentration in the effluent was around 11 mg VSS L^{-1}, corresponding to a sludge retention time of 90 d. The average diameter of the granules remained approximately constant.

Despite the system was stable and the limiting substrate (nitrite) was almost completely consumed (Figure 5.7), the nitrogen removal rate was

relatively low (0.08 g N $(L\ d)^{-1}$) because of the decrease of the SAA. Thus, the Anammox unit ASBR2 was the limiting step of the process. If a comparison is done, the NLR obtained in the previous work (Section 5.4.2) at 20 ºC was about 4 times higher. Furthermore, the NLR reported by Isaka *et al.* (2007) and Cema *et al.* (2007), working at 20 ºC and 17 ºC, respectively, were even much higher. However, all these systems had biomass concentrations much higher than the concentration in ASBR2 (1.5 g VSS L^{-1}). Actually, if the comparison between ASBR1 and ASBR2 is made in terms of SAA, the SAA observed for the biomass in ASBR2 was higher. Therefore, if a moderate to high NLR has to be removed at low temperature, it would be necessary to have a higher concentration of biomass in the reactor and a very good biomass retention system.

5.5. CONCLUSIONS

The possibility of operation of Anammox systems at relatively low temperatures (about 18-20 ºC) has been proved, despite the fact that the optimum has been found at 35-40 ºC. Two systems treating synthetic and partially nitrified sludge digester effluents were successfully operated maintaining their stability for more than one month.

In order to achieve this stable operation, the slow and gradual decrease of the temperature of operation has been revealed as a suitable strategy which led to an adaptation of the biomass. Due to the decrease in the SAA of the biomass, higher concentrations of Anammox sludge would be necessary in order to remove relatively high NLR at low temperatures. Besides, capacity calculations using SAA have been demonstrated to be useful in order to control the operation and prevent overloads.

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Chapter 6

Evaluation of a deammonification system by specific Anammox activity measurements

ABSTRACT. Response surface models were successfully applied to evaluate the performance of the Anammox process in a deammonification system (i.e. one-stage biofilm Anammox process).

Specific Anammox activity was measured by a manometric method and employed as the response variable. Temperature, pH and the concentrations of substrates were chosen as the controlled variables.

The models pointed out that the significant controlled variables were the temperature, the value of pH and the ratio between the unionized species of the substrates (free ammonia and free nitrous acid). There were interactions among them caused by chemical equilibriums. Total nitrogen concentration and ammonium concentration were found to be not significant in the tested range.

The optimum values of temperature, pH and free ammonia to free nitrous acid ratio within the test ranges were, respectively, 30 ˚C, 7.0 and 0.3.

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6.1. INTRODUCTION

Several Anammox full-scale plants are in operation (Chapter 1, Section 1.8.2). All of them have reached their design capacity treating wastewater from different origins which indicates the wide applicability of the process. It is important to point out that the time for the first full-scale reactor start-up was about 3 years while the newer ones were started up in few months (Abma *et al.*, 2007a; Abma *et al.*, 2007b; Wett, 2007; Joss *et al.*, 2009; SYVAB, 2009). This fact was mainly due to a better knowledge about Anammox process and a greater availability of inoculum. Under the denomination of deammonification (DEMON), there are two full scale plants in Austria (500 m^3, 300 kg N d^{-1}) and Switzerland (400 m^3, 250 kg N d^{-1}) treating the supernatant of sludge digesters (Wett, 2006; 2007). The start up time of the first plant was 2.5 years, while the plant located in Switzerland, inoculated with sludge from the first one, reached its design load in only 50 days.

In Sweden, the implementation of the Anammox process is being studied at pilot scale in Hammarby Sjöstad Research Station (Stockholm). The pilot plant is carrying out the autotrophic nitrogen removal in one single unit (deammonification), which comprises partial nitrification and Anammox, coexisting in one biofilm attached to Kaldnes plastic carriers (Szatkowska *et al.*, 2007). This plant was operated at an NLR of about 0.2 g (NH_4^+-N + NO_2^--N) (L $d)^{-1}$ and its nitrogen removal efficiency was around 30-40%. Previous results may indicate that the relatively low efficiency could be improved by operating the plant at the optimum conditions which maximize the Anammox rate.

Along the previous chapters and also in many published works, some of the variables influencing the Anammox process were studied, like inhibition by substrates (Chapter 3), the effects of the temperature (Chapter 5) or the effects of pH (Strous *et al.*, 1999). However, in all these cases, each studied variable was considered separately. Thus, if

interactions among variables (i.e. cumulative effects) are present, they could not be easily observed. Furthermore, it would also be important to know what variables are the most important. This also implies the research about the effects of several variables at the same time. According to the literature, the following variables are expected to be the most important:

Temperature: This variable exerts a very important influence on the Anammox activity (Szatkowska and Plaza, 2006; Dosta *et al.*, 2008). According to some authors, Anammox activity can be detected and measured from below 10 ˚C (Rysgaard *et al.*, 2004) to 40 ˚C. Several authors (Strous *et al.*, 1999; Egli *et al.*, 2001; Toh *et al.*, 2002; Yang *et al.*, 2006) found that optimum temperature for the operation of the Anammox process was between 30 ºC and 40 ºC.

pH: Strous *et al.* (1999) determined a physiological pH range for Anammox between 6.7 and 8.3. A later work (Egli *et al.*, 2001) agreed about the lower boundary, but reported a higher upper limit, with Anammox activity detected at pH 9.0. Furthermore, they observed the optimum between 7.5 and 8.0. Fux *et al.* (2004) reported a total drop of the Anammox activity from a pH value of 9.3.

Concentrations of substrates: The inhibitory effects caused by nitrite on the Anammox process are well known (Strous *et al.*, 1999; Dapena-Mora *et al.*, 2007), but the different authors did not agree about the concentration level that should not be exceeded (50-150 mg N L^{-1}, Strous *et al.*, 1999; 30-50 mg N L^{-1}, Fux *et al.*, 2004). Dapena-Mora *et al.* (2007) and the work presented in Chapter 3 also reported inhibitory effects caused by ammonium. Specifically, Dapena-Mora *et al.* (2007) reported a 50% inhibition concentration of 55 mM NH_4^+. Besides, it is suspected (Chapter 3) that the inhibitors can be the unionized forms of the substrates: Free Ammonia (FA) and Free Nitrous Acid (FNA), similarly to the case of nitrifiers (Anthonisen *et al.*, 1976). The value of pH affects to

the distribution of ionized and free forms of ammonia and nitrous acid, according to the following chemical equilibriums:

$$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O \quad (6.1)$$

$$NO_2^- + H_3O^+ \leftrightarrow HNO_2 + H_2O \quad (6.2)$$

FA and FNA concentrations can have more important effects than the caused by the value of pH. Thus, in that case, the relationship FA/FNA was used as the controlled variable and its value was calculated with the equations provided by Anthonisen *et al.* (1976).

To study the effect of the above indicated variables, Response Surface Methodology (RSM), which is a type of multivariate data analysis, can be useful to find the relationships between several controlled variables and one or more response variables (Box and Wilson, 1951). When three generic variables are considered, the standard RSM equation is the following:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (6.3)$$

Where, Y is the dependent variable or response; x_1, x_2 and x_3 are the independent (controlled) variables; b_0 is the regression coefficient at centre point; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second order coefficients; and b_{11}, b_{22}, b_{33} are quadratic coefficients (Seth and Chand, 2000; Chang *et al.*, 2006). When the values of the independent variables are coded (dimensionless) the coefficients have the same dimensions and, subsequently, the same units, of the dependent variable.

Dapena-Mora *et al.* (2004) have reported that batch specific Anammox activity measurements are useful in order to monitor the process and to assess its long term performance. Thus specific activity can be considered

as an interesting response variable to optimize the performance of reactors which involve the Anammox process. Moreover, previous works (Bator, 2006; Gut *et al.*, 2007) demonstrated that multivariate data analysis can be successfully applied for the evaluation of the deammonification process. However, in those cases the overall performance of the combined partial nitrification-Anammox system was studied, while in the present work the focus is on the Anammox step.

6.2. OBJECTIVE

The objective of this work was to look for the optimum values of the variables which mainly influence the Anammox process. Response surface models will be applied to accomplish this objective.

6.3. MATERIALS AND METHODS

6.3.1. Reactor and biomass characteristics

Biomass used to carry the different assays was collected from a deammonification reactor of 22 L which was installed at Hammarby Sjöstad Research Station, (Stockholm, Sweden) (Figure 6.1).

The reactor was filled (40%) with Kaldnes K1 rings with a diameter of 10 mm and a length of 7 mm (Figure 6.2). Their specific surface area, when they are completely packed, is 500 m^2 m^{-3}. The reactor was fed with supernatant of an anaerobic digester with an ammonium concentration of 600-750 mg N L^{-1}. The hydraulic retention time was 3 days and the temperature of operation was not controlled and ranged between 14 and 20 ºC. The specific Anammox activity (SAA) of the biomass was 1.1 g N_2-N $(m^2\ d)^{-1}$ (measured at 30 ºC, pH 7.8, 70 mg NH_4^+-N L^{-1}, 70 mg NO_2^--N L^{-1}).

Figure 6.1. Deammonification reactor (centre, indicated with an arrow; picture by Sandra Martínez and Zaira Hernando).

Figure 6.2. Vessel prepared to perform a SAA test with Kaldnes K1 rings (picture by Sandra Martínez and Zaira Hernando).

6.3.2. Specific Anammox activity tests

Batch experiments to determine the SAA were systematically performed according to the methodology described in Chapter 2. Since the method was developed for suspended or granular biomass, some modifications were done on the procedure due to the use of biofilm biomass on Kaldnes K1 plastic carriers. Therefore, in this work, instead of using the measurement of the amount of biomass in terms of suspended solids, the SAA was referred to the biofilm area. A fixed number of 15 Kaldnes rings was employed in each individual test. The amount of biomass in these 15 rings was estimated about 0.2 g VSS (17 g VSS m^{-2}). They were gently washed with the buffer, avoiding detachment of the biofilm. Later they were introduced in the vials where tests were done (Figure 6.2). SAA was finally calculated as the nitrogen removed from the liquid phase per unit of time and biofilm area.

6.3.3. Statistic methods, experimental strategy and computer data analysis

Factorial designs with 3 levels (low, centre and high) for each controlled variable were employed. These levels were represented by -1, 0 and 1. Three controlled variables were chosen for each of the two studies. In the first one they were: ammonium concentration, pH and temperature; and in the second study: total nitrogen, FA to FNA ratio and temperature.

Two experimental designs were employed, Box-Behnken (Box and Behnken, 1960) for the first study and Central Composite Face Centered (CCFC) (Box and Wilson, 1951) for the second study. Both designs can fit quadratic models (i.e. models with squared terms and products of two factors) and employ three levels (values) per variable. However, Box-Behnken designs can be expected to have poor prediction abilities at the corners of the cube or hypercube that encloses the design (NIST/SEMATECH, 2010). Since the results of the first study pointed out that the optimum was at one corner of the mentioned cube (Section 6.4.1), it was decided to use a CCFC design for the second study.

6.3.3.1. First study

In the first study, a Box-Behnken design was done. The number of experiments for this design is 12 plus the central point replicates (NIST/SEMATECH, 2010). In this case, the 12 individual experiments located at the corners of the design were performed by duplicate and, besides, six replicates of the central point were done. Replicates were necessary in order to assess the reproducibility of the experimental measurements and to reduce their dispersion. Therefore, 30 experiments were performed.

A fixed nitrite concentration of 70 mg NO_2^--N L^{-1} was employed, according to Dapena-Mora *et al.* (2007). The selected high level of the ammonium concentration (Table 6.1) was representative of the concentration in the feeding of the reactor. The low level is the stoichiometric concentration (substrates molar ratio about 1.3, according to Strous *et al.*, 1999) corresponding to the nitrite concentration of the tests.

Table 6.1. Values corresponding to the coded levels of the controlled variables, first study.

	-1	**0**	**1**
T (°C)	15.0	22.5	30.0
NH_4^+-N (mg N L^{-1})	53	300	530
pH	7.0	7.8	8.6

Values of pH lower than 7.0 were not considered because, despite the process can be carried out at these values, they are usually slightly higher than 8 in the influent of the reactor. In the case of temperature (T), the upper boundary of the test range was conservative taking into account previous works (Dosta *et al.*, 2008). However, the feeding of the deammonification reactor was usually under 20 ºC because the location of this reactor was far from the anaerobic digester. It was estimated that,

improving the isolation of the system, it would be possible to obtain a temperature not higher than 30 ºC.

6.3.3.2. Second study

Since it was observed that the reproducibility of the experimental data from the first study was good, which means that the experimental errors were not significant, the CCFC design was done without replicates of the experiments not located in the central point (14 runs) and the number of replicates of the central point was reduced to 3 instead of 6. Therefore, the total number of experiments of this study was 17.

The selected ranges of the controlled variables can be seen in Table 6.2. The two substrates were supplied in equimolar ratio; therefore, in the experiments with the lower TN concentration, initial nitrite and ammonium concentrations were 50 mg N L^{-1} of each compound. These concentrations were considered enough to avoid diffusional substrate limitations during the experiments. A nonlinear scale of FA/FNA ratio was selected in order to have a wider range. The low level was 0.3 and the high level was 3000, with the central point at 30. The range was transformed by logarithms to introduce it into the program. To manipulate the FA/FNA ratio in the medium, the appropriate pH was established in each individual test by adding the necessary volumes of diluted acid or base (calculations based on Anthonisen *et al.*, 1976; pH range employed: 6-8.3). Thus, in this case pH is considered as a dependent controlled variable, because its value in each case was determined by the values of FA/FNA ratio and T.

Table 6.2. Experimental conditions, second study.

	-1	**0**	**1**
T (°C)	15.0	22.5	30.0
TN (mg (NH_4^+-N+NO_2^--N) L^{-1})	100	300	500
NH_4^+-N (mg L^{-1})	50	150	250
NO_2^--N (mg L^{-1})	50	150	250
FA/FNA	0.3	30	3000
log FA/FNA	-0.52	1.48	3.48

6.3.3.3. Computer data analysis

To analyze the results obtained, the program MODDE, developed by Umetrics AB was used (Eriksson *et al.*, 2001). The method employed to fit the models was the Multiple Linear Regression (MLR).

Once the experimental values of SAA were introduced in MODDE, the program identified the outliers. An outlying observation, or outlier, is a value that appears to deviate markedly from other members of the sample in which it occurs (Grubbs, 1969). The program assumes a normal distribution of the residuals (differences between the observed and the predicted values) and considers as outliers the diverging points whose residuals are larger than 4 standard deviations. In any of the two studies the detected outliers were discarded and then the model was fitted to the experimental data.

6.4. RESULTS AND DISCUSSION

6.4.1. First study: Effects of ammonium, pH and temperature

The 30 individual experiments were performed (Table 6.3) and then the values of SAA were introduced in MODDE. The program identified the outliers. In this case, the program detected that the individual tests 18, 19 and 24 were probable outliers, so they were not considered to do the statistical calculations.

Table 6.3. First study, test plan and results. N is the number of each individual test, controlled variables are coded and the response (SAA) is given in g N_2-N $(m^2\ d)^{-1}$.

N	1	2	3	4	5	6
T	-1	-1	-1	-1	-1	-1
NH_4^+-N	-1	-1	0	0	0	0
pH	0	0	-1	-1	1	1
SAA	0.103	0.052	0.082	0.124	0.057	0.095

N	7	8	9	10	11	12
T	-1	-1	0	0	0	0
NH_4^+-N	1	1	-1	-1	-1	-1
pH	0	0	-1	-1	1	1
SAA	0.057	0.139	0.837	0.639	0.481	0.342

N	13	14	15	16	17	18
T	0	0	0	0	1	1
NH_4^+-N	1	1	1	1	-1	-1
pH	-1	-1	1	1	0	0
SAA	0.568	0.800	0.502	0.558	0.836	1.33

N	19	20	21	22	23	24
T	1	1	1	1	1	1
NH_4^+-N	0	0	0	0	1	1
pH	-1	-1	1	1	0	0
SAA	1.38	0.869	0.492	0.543	0.835	1.33

N	25	26	27	28	29	30
T	0	0	0	0	0	0
NH_4^+-N	0	0	0	0	0	0
pH	0	0	0	0	0	0
SAA	0.594	0.567	0.600	0.453	0.395	0.591

The model was fitted by MLR and the quality of the fit was good, with 94% of the variability of the response explained by the model (i.e. R^2 = 0.94) and 85% of that variability can be predicted by the model (i.e. Q^2 = 0.85). The significant first order variables (Figure 6.3) were T and pH while the effect of the initial concentration of ammonium was not significant in the range employed. The interaction between T and pH and the quadratic term of T were also significant. In all these cases, the confidence interval of the coefficients did not cross zero.

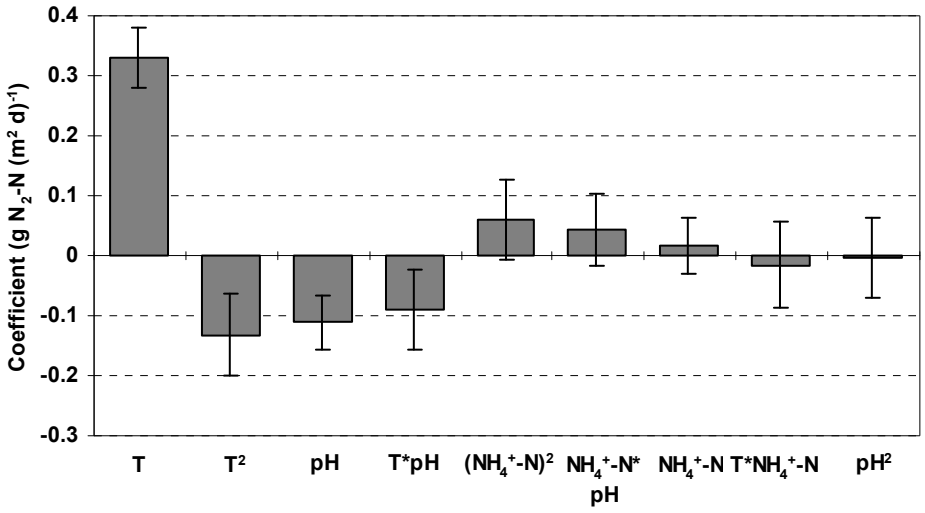

Figure 6.3. Scaled and centred model coefficients with 95% confidence interval (i.e. there is a 95% probability that the true value lies in this interval) (g N_2-N (m^2 d)$^{-1}$).

Fixing the ammonium concentration in the central point, a three-dimensional surface plot (Figure 6.4) could be drawn in order to represent the behaviour in a graphical way. According to this model, a temperature of 30 ºC and a pH value of 7 are the optimum conditions to operate the reactor within the tested conditions.

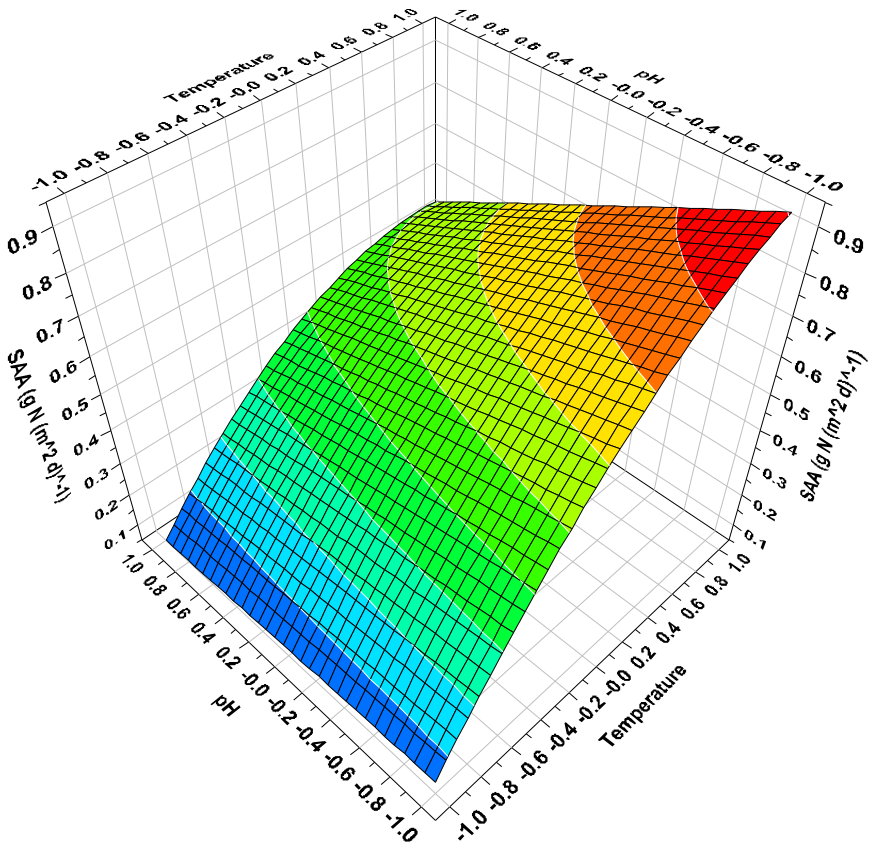

Figure 6.4. Three-dimensional surface plot, SAA in g N_2-N $(m^2\ d)^{-1}$, pH and T as coded variables, ammonium concentration fixed at the central point (300 mg NH_4^+-N L^{-1}).

The model allows calculating the activity change caused by one variable with the two others fixed. When focus is on T, it can be observed (Figure 6.5a) that at the lower part of the T range; a small change in this variable will produce an important change in the SAA. However, on the upper part of the T range, the increase of SAA caused by T is not as important. The observed behaviour would not be the expected one, taking into account that the dependency reported in literature is Arrhenius-type (Strous *et al.*, 1999; Dosta *et al.*, 2008). This fact might be attributed to the different

conditions employed by other authors when the influence of the temperature was researched.

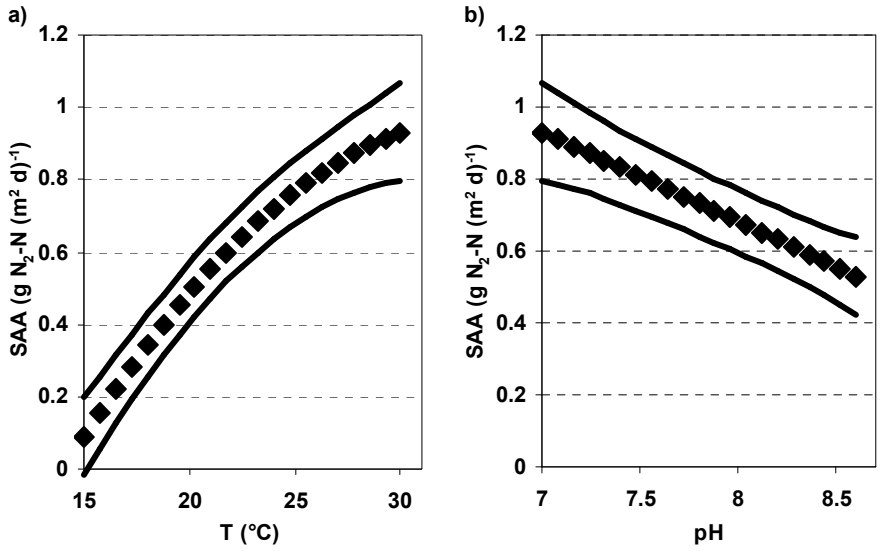

Figure 6.5. a) SAA as a function of T at pH 7 and NH_4^+-N at the central point (300 mg NH_4^+-N L^{-1}). b) SAA as a function of pH at 30 ˚C and NH_4^+-N at the central point (300 mg NH_4^+-N L^{-1}). The continuous lines represent the 95% confidence interval (i.e. there is a 95% probability that the true value lies in this interval).

In the case of the effect of the pH, the behaviour is linear (Figure 6.5b) and the SAA was decreasing with the increase of the pH value. This dependency does not agree with the reported by Strous (2000). This author reported an increase of the Anammox activity with the increase of the pH value from 7 to 8 and then a decrease from pH 8 to 9.

6.4.2. Second study: Effects of total nitrogen, FA/FNA ratio and temperature

The 17 individual experiments were performed (Table 6.4) and the obtained values of SAA were analyzed by means of MODDE program which detected that the individual test 10 was a probable outlier. The model was fitted by MLR with a good quality of the fit. About 99% of the variability of

the response was explained (i.e. R^2 = 0.99) and 92% of the variability was predicted by the model (i.e. Q^2 = 0.92).

Table 6.4. Second study, test plan and results. N is the number of each individual test, controlled variables are coded and the response (SAA) is given in g N_2-N $(m^2\ d)^{-1}$.

N	1	2	3	4	5	6
T	-1	-1	-1	-1	-1	0
TN	-1	-1	0	1	1	-1
log FA/FNA	-1	1	0	-1	1	0
SAA	0.202	0.067	0.034	0.147	0.096	0.627

N	7	8	9	10	11	12
T	0	0	0	1	1	1
TN	0	0	1	-1	-1	0
log FA/FNA	-1	1	0	-1	1	0
SAA	1.28	0.436	0.618	1.42	1.38	1.72

N	13	14	15	16	17
T	1	1	0	0	0
TN	1	1	0	0	0
log FA/FNA	-1	1	0	0	0
SAA	2.26	1.24	0.618	0.630	0.702

The individual significant variables (Figure 6.6) were T and the ratio FA/FNA. The influence of TN was not significant. This also means that the influence of the absolute values of FA and FNA was not significant, taking into account that, for the same ratio FA/FNA, the absolute values of the unionized compounds increase with the increase of TN. The highest concentration of FA tested was about 17 mg NH_3-N L^{-1}, while the highest concentration of FNA was about 0.6 mg HNO_2-N L^{-1}. The results for FA agree with Chapter 3. According to this chapter, no significant effects may be expected at 17 mg NH_3-N L^{-1}. In the case of FNA, the biomass tested in the present work was significantly more resistant than the biomass

employed for the tests reported in Chapter 3. Apart from that, the interaction between T and the ratio FA/FNA was significant, as well as the quadratic terms of T and FA/FNA.

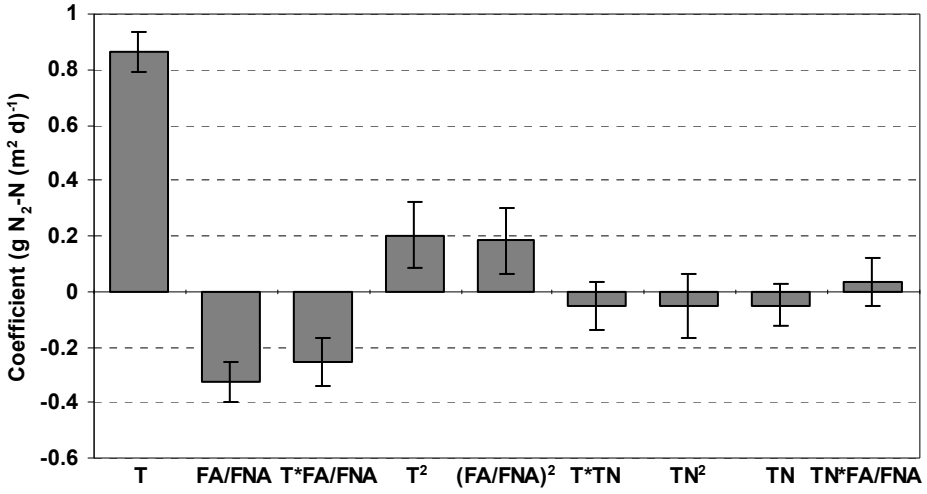

Figure 6.6. Scaled and centred model coefficients with 95% confidence interval (i.e. there is a 95% probability that the true value lies in this interval) (g N_2-N $(m^2\ d)^{-1}$).

Since TN is not significant, a three-dimensional surface plot can be built, employing as controlled variables T and FA/FNA (Figure 6.7). According to this, the operation of the reactor at 30 ˚C and with FA/FNA ratio of 0.3 will give the highest value of SAA.

In this case, taking into account the analysis of the variation in the response from the optimum point (Figure 6.8), the most important variations will be observed close to that point. Since both quadratic terms were found to be significant, the relationships SAA/T and (FA/FNA)/T are not a straight line.

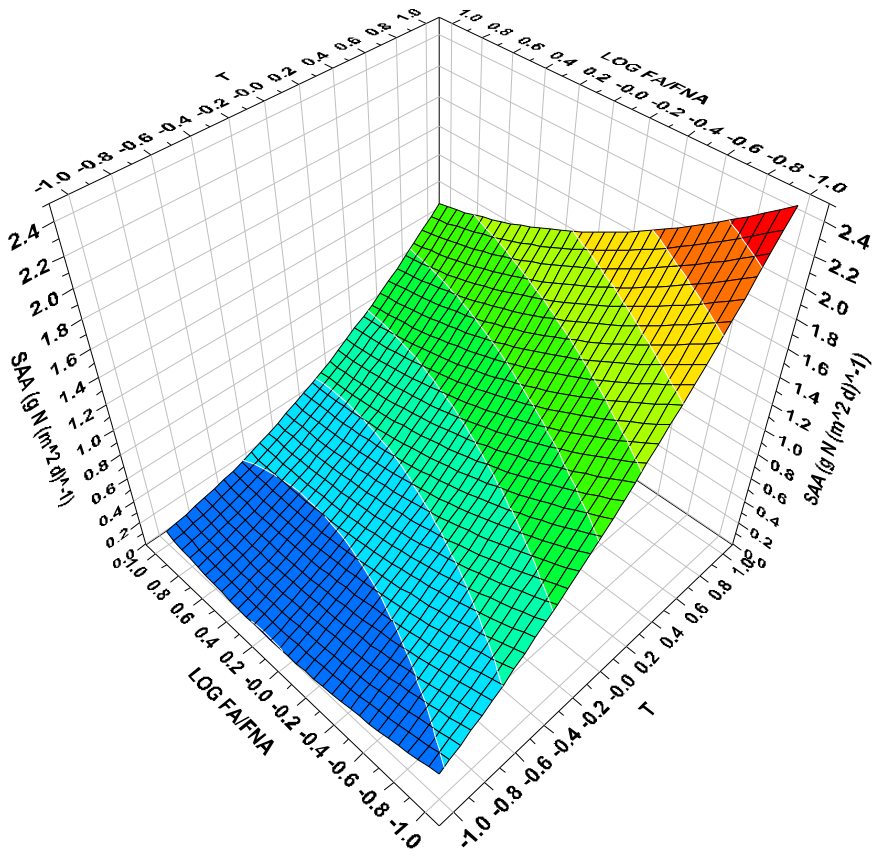

Figure 6.7. Three-dimensional surface plot. SAA in g N_2-N $(m^2\ d)^{-1}$, log FA/FNA and T in form of coded variables, TN fixed at the central point (300 mg (NH_4^+-N+NO_2^--N) L^{-1}).

The trend of SAA with the change on T is different from the observed in the previous experience (Section 6.4.2) and in this case the dependency agrees well with an Arrhenius model. Therefore, if these data are fitted to this kind of model, the activation energy obtained would be 84.7 kJ mol^{-1}, which is close to the values obtained by Strous *et al.* (1999) and Dosta *et al.* (2008).

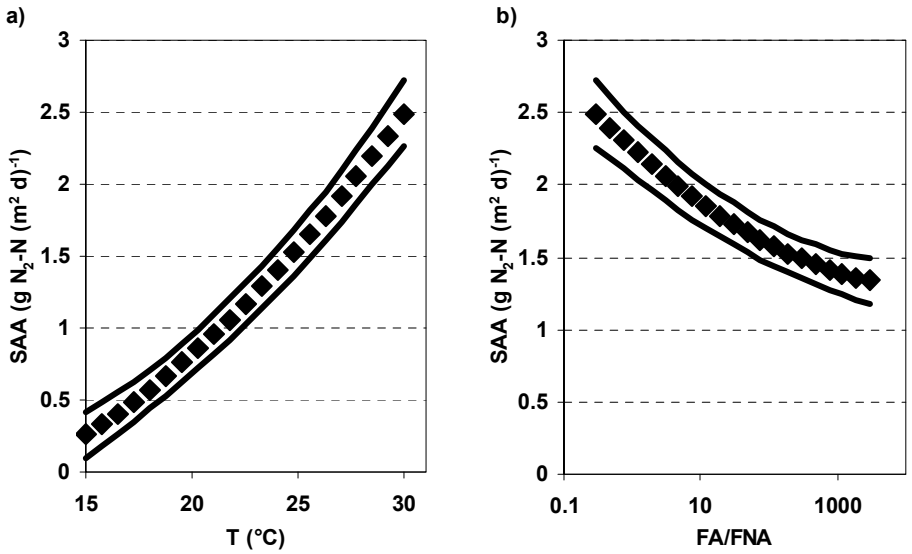

Figure 6.8. a) SAA as a function of T with FA/FNA ratio fixed at 0.3 and TN at the central point (300 mg (NH_4^+-N+NO_2^--N) L^{-1}). b) SAA as a function of FA/FNA at 30 °C and TN at the central point (300 mg (NH_4^+-N+NO_2^--N) L^{-1}). The horizontal axis in this figure is in logarithmic scale. The continuous lines represent the 95% confidence interval (i.e. there is a 95% probability that the true value lies in this interval).

6.4.3. Application

Models from the two studies show that T is the most important variable and that the process should be operated at the highest possible temperature (within the range). This fact agrees with what was reported by Szatkowska *et al.* (2006) and Dosta *et al.* (2008). Taking into account that during winter the reactor is operating very closely to the lowest tested T, it will be interesting to reduce the loss of energy and to raise the temperature. According to the model, pH values about 7 and FA/FNA ratios near to 0.3 are the optimum. The work may confirm that there is a influence on SAA caused by the unionized substrates. Inhibition by FA and FNA is also pointed out and discussed by Fernández *et al.* (2008) and in Chapter 3. Within the tested conditions, FNA inhibition was not as strong as the one reported by Jung *et al.* (2007) and Fernández *et al.* (2008). These authors observed significant long-term inhibition in the presence of

FNA concentrations as low as 0.8-1.2 μg HNO_2-N L^{-1} (calculated from their data) and 0.5 μg HNO_2-N L^{-1}, respectively. However in the present work the SAA obtained was high (2.26 g N_2-N $(m^2\ d)^{-1}$) working at concentrations of FNA up to 0.6 mg HNO_2-N L^{-1}, which are the ones corresponding to the tests with FA/FNA at 0.3, TN 500 mg (NH_4^+-N+NO_2^--N) L^{-1} and T 30 ºC. One hypothetical explanation to this fact can be that the outer layer of the biofilm is able to protect the inner part where the Anammox bacteria will be located, at least during short-term exposure. This stronger resistance to changes in the environmental conditions has been observed in experiments with nitrifying biofilm (Olem and Unz, 1980) and ferro-oxidizing biofilm (Karamanev and Nikolov, 1988).

When working at the same commented conditions of TN and T (500 mg (NH_4^+-N+NO_2^--N) L^{-1} and 30 ºC), but FA/FNA at the highest level (3000, with FA 17 mg NH_3-N L^{-1}), the SAA obtained was 1.24 g N_2-N $(m^2\ d)^{-1}$. This means that FA inhibition might be more important than FNA inhibition in this case. Especially since it was unlikely that substrate limitation took place in the conditions of the test (FNA 6 μg HNO_2-N L^{-1}).

In order to apply this research to the control of one industrial-size reactor, it is clear that the important variables are T, pH and the concentrations of unionized substrates. When the influent was previously treated in an anaerobic digester working at the mesophillic range, it would be strongly advisable to have a good isolation system in order to keep the temperature of the wastewater near the optimum range for the Anammox process. Actually, this strategy was implemented in the deammonification plant and it led to a slow efficiency increase along time. Besides, a control system involving substrates concentrations and pH would be applied by means of on line selective electrodes of nitrite, ammonium and pH. The control system will change the flow of oxygen in order to keep the nitrite to ammonium rate close to the stoichiometric one. Then, the values of concentrations and the pH in the reactor would allow calculating the FNA and FA concentrations at any time. According to these data, strategies for

pH change (acid/base addition; control of the recirculation flow rate...) allow operating within the optimum range of FA/FNA ratio.

6.5. CONCLUSIONS

The performed studies proved that response surface modelling is able to provide useful models for the Anammox process.

Temperature, pH value and the FA/FNA ratio are pointed out by the models as the most important variables among the ones tested. Optimum values within the tested ranges were obtained.

The knowledge about the most important variables involved would allow the development of a control strategy for an industrial-size reactor which can be able to optimize its operation.

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Chapter 7

Influence of the shear stress and salinity on the Anammox biofilm formation

ABSTRACT. Anammox biomass has a relatively long duplication time and low yield. This is an advantage because the production of surplus sludge is reduced; however it also implies that the process must be operated in reactors with very good sludge retention. Some of the systems used to improve biomass retention are based on biofilm reactors; therefore it would be important to research the ability of Anammox biomass to form biofilms under different conditions.

The effects of shear stress and salinity (NaCl and $CaCl_2$) on Anammox biofilm formation were studied by means of a sensor based on vibration properties. Anammox bacteria showed good attachment capacity, with an initial adhesion phase lasting for 5-7 days at the three different flow rates tested (25.2; 8.4; 7.3 L h^{-1}), corresponding to Reynolds numbers 188, 63 and 54. Besides, the mechanical stability of the biofilm appeared to be better when it was formed under higher shear stress.

The presence of the two salts favoured the formation of Anammox biofilm because of reduction of electrostatic repulsion forces. The effects of the $CaCl_2$ were stronger than those caused by NaCl probably because divalent cationic bridging was taking part when the calcium salt was used. Incorporation of inorganics into the biofilm was observed in both cases.

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[1] Some parts of this chapter have been included in: **Fernández I., Pereira A., Melo L.F., Mosquera-Corral A., Campos J.L., and Méndez R.** Influence of shear stress and salinity on Anammox biofilm formation. *In preparation.*

7.1. INTRODUCTION

Anammox biomass is characterized by its long duplication time and low biomass yield (Strous *et al.*, 1999). Therefore, the improvement of the Anammox biomass retention is one of the keys to achieve the worldwide full scale application. Systems with good biomass retention can reduce the duration of the start up period and provide better operational conditions. Anammox biomass grows as a biofilm in some of the successfully employed reactors, either on moving bed carriers (Helmer *et al.*, 2001; Gaul *et al.*, 2005; Szatowska *et al.*, 2007) or on a fixed bed (Tal *et al.*, 2006; Isaka *et al.*, 2007; Liu *et al.*, 2009).

The formation of bacterial biofilms must, necessarily, begin with the adhesion of a small number of bacterial cells to a surface (Costerton, 1999). Microbial adhesion has been described in the literature by means of Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, developed by Derjaguin and Landau (1941) and Verwey and Overbeek (1948). According to this theory, the net interaction force arises from the balance between attraction forces (van der Waals type) and electrostatic double-layer forces, which are in general repulsive. The increase of the ionic strength of the liquid medium can reduce this electrostatic repulsion by compressing the double layer, thus an increase of the salinity can favour the biofilm formation.

Apart from DLVO forces, other interactions can play a role in the adhesion process, like hydrophobic interactions in aqueous medium. Moreover, bacteria are capable of producing and excreting polymeric metabolites. Extending chains of polymers attached to cells can interact with the surface, holding the cells near the liquid-solid interface (polymer bridging) and favouring the adhesion (Characklis and Cooksey, 1983). The existence and properties of external appendages can also have a strong influence in the adhesion and growth of the biofilm. Therefore, the rate of

adhesion depends not only of the surface type but also of the ability of the bacteria (or consortium) to attach.

An important factor to be considered is the fluid motion, which can have two opposite effects on biofilm formation. An increase in fluid velocity increases the shear stress exerted on the deposited microorganisms and this can cause the detachment and consequently the decrease of bacterial activity, when microorganisms are not highly resistant to shear stress (for example Anammox organisms; Arrojo *et al.*, 2006). On the other hand, higher fluid velocities reduce mass transfer resistance between the bulk liquid and the biofilm, thus a higher biofilm growth can also occur.

The presence of inorganic ions may also affect microbial attachment to surfaces. It has been reported by Fernández *et al.* (2008) that high concentrations of NaCl can promote the granulation of Anammox biomass, which can be considered as a form of biofilm without support material. These authors also reported that Anammox organisms are relatively resistant to high salinity and NaCl concentrations up to 10 g L^{-1} were not significantly affecting Anammox activity. Besides, Kartal *et al.* (2006) obtained similar results and they also reported that freshwater Anammox bacteria could adapt to NaCl concentrations as high as 30 g L^{-1}.

About the effects caused by the presence of divalent cations like Ca^{2+}, they are well known as promoters of anaerobic biomass granulation (Tiwari *et al.*, 2006). Divalent cations can form bridges between negatively charged groups on cell surfaces and the support material and they can also link exo-cellular polymers (Schmidt and Ahring, 1994; Hulsoff Pol *et al.*, 2004).

The use of dynamical methods to study bacterial biofilm formation allows the observation of the adhesion phase. The maximum adhesion capacity of each bacterial strain is related with the ability of the cells to form a biofilm (Cerca *et al.*, 2004). Moreover, the results of the dynamical studies could be employed in order to develop a model useful to derive

predictions about the growth of the biofilm (Melo and Vieira, 1999). Finally, the knowledge about the formation and properties of Anammox biofilms could be valuable in the design and operation of attached biomass reactors.

7.2. OBJECTIVES

The objective was to study the ability of the Anammox biomass to form biofilms under different conditions of shear stress (i.e. flow speed) and under presence of salts.

7.3. MATERIALS AND METHODS

7.3.1. Mechatronic surface sensor monitor

In order to monitor the growth of the biofilm along time, a Mechatronic Surface Sensor (MSS) was employed (Pereira *et al.*, 2006). The working principle of the MSS relies on the fact that the attachment, growth and detachment of biomass on a support can be studied measuring the changes on the surface waves applied through the biofilm.

The sensor (Figure 7.1) was composed of a polyvinyl chloride (PVC) plate that closed the open base of a semi-circular conduct made of poly(methyl methacrylate) (PMMA). This conduct was part of a flow cell where the sensor was inserted. Two electronic instruments were glued on the outer surface of the PVC plate, a piezoelectric actuator and a vibration sensor (accelerometer) (Figure 7.2). The first one generated the surface waves which propagated along the plate. The accelerometer was glued on the opposite part of the plate in order to measure the characteristics of the waves after they propagated through the plate. From this data, the associated software (developed in LabVIEW®) assessed all the wave parameters that can be correlated with the biofilm build-up, in particular the variation of the amplitude.

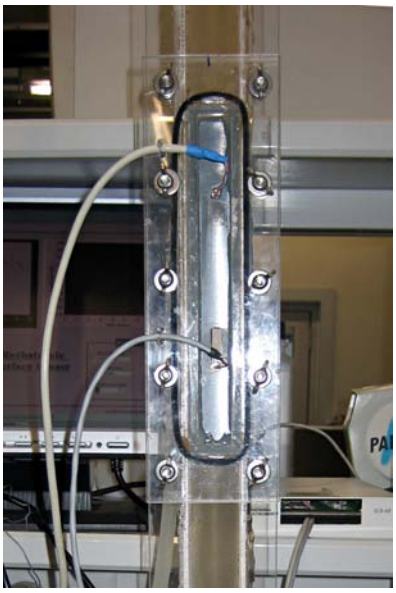

Figure 7.1. The MSS sensor inserted in a flow cell.

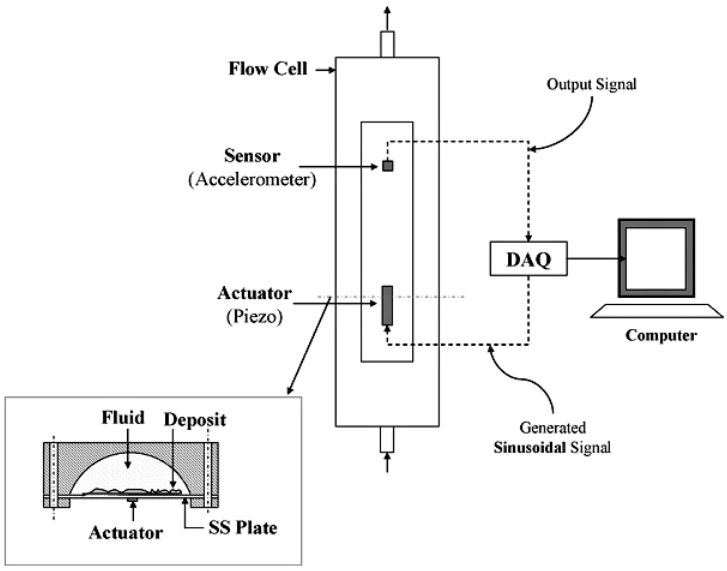

Figure 7.2. Schematic of the MSS sensor: the deposit in this case is the biofilm. DAQ is the Data Acquisition system. (Adapted from Pereira *et al.*, 2009).

The MSS device was inserted into a semi-cylindrical flow cell (internal diameter = 30 mm; equivalent hydraulic diameter = 18.3 mm), similar to the one described by Pereira *et al.* (2002). The flow cell had a height of 107 cm and an internal volume about 380 mL. It contained 6 removable sample collectors with a PVC plate (inner side) of about 1.9 cm^2 in order to take periodical samples of the biofilm. The specific mass of biofilm was periodically measured in terms of Volatile Suspended Solids (VSS) per unit area by mechanically removing the biofilm from the PVC plates. A calibration of the MSS was performed for each experiment in order to find the relationship between the specific mass of biofilm and the amplitude of the vibration measured by the sensor. This relationship was assumed to be linear according to Pereira *et al.* (2008).

A shielded input-output connector block (SCB-68, National Instruments), a noise rejecting shielded cable (SHC68-68-EPM, National Instruments) and a data acquisition board (NI-PCI 6221, National Instruments) were employed to acquire the signal and transmit it to the personal computer. The signal was appropriately processed by the software (developed in LabVIEW®). During the present work, the MSS performed one measurement per hour.

In order to reduce the noise of the signal, two statistical tools were employed. First, the 24 measurements corresponding to each day were used to calculate a daily average. The second step was to use a weighted moving average (WMA). Five points were used in each case to calculate the average, two before and two after the central point, which had twice the value of each other four points, according to Equation 7.1:

$$WMA_i = (y_{i-2} + y_{i-1} + 2y_i + y_{i+1} + y_{i+2})/6 \quad (7.1)$$

Where WMA_i is the value of the moving average and y_{i-2} to y_{i+2} are the five points considered, two after and two before y_i.

7.3.2. Reactor and inoculum

To study the biofilm formation in the flow cell, an external Sequencing Batch Reactor of about 1 L of working volume was employed. The reaction medium was pumped from the bottom of the reactor and impelled by a positive displacement pump (Seko Tekna AXL 602) to the bottom of the flow cell. Then, it was returned to the reactor.

The SBR was operated in cycles of 6 hours distributed in four periods: mixed fill (300 min), mix (30 min), settle (15 min) and draw (15 min) according to Dapena-Mora *et al.* (2004a). The positive displacement pump which impelled the liquid through the flow cell was only connected during the first two periods. The control of the different periods of the operational cycle was performed using a PLC system (CPU224, Siemens). Two peristaltic pumps were employed to feed and draw the unit. Temperature was controlled at 30 ºC by using a thermostatic system. pH was not controlled and ranged between 7 and 8. The hydraulic retention time of the whole system (reactor plus flow cell) was about 1.2 d.

The reactor was initially inoculated with enriched Anammox sludge taken from a laboratory scale SBR (Dapena-Mora *et al.*, 2004b). The initial concentration of biomass was around 0.3 g VSS L^{-1} and its SAA was 0.22 g N (g VSS $d)^{-1}$.

The system was fed with a synthetic autotrophic medium described in Section 2.4 (Dapena-Mora *et al.*, 2004b). The nitrogen loading rate applied was about 0.06 g(NH_4^+-N + NO_2^--N) (L $d)^{-1}$ and the ammonium to nitrite molar ratio was approximately stoichiometric (1.3). During the experiments the system was operating with an overall nitrogen removal higher than 90%.

7.3.3. Experimental strategy

A series of experiments were performed in order to research the influence of the flow rate and the salinity of the medium (Table 7.1).

Three different flow rates were tested. The lowest flow rate was near to the minimum necessary to maintain the fluidized conditions of the biomass through the cell. The highest was chosen in order to avoid shear stress inhibition (Arrojo *et al.*, 2006).

Regarding salinity tests, two salts were employed, NaCl and $CaCl_2$. Fernández *et al.* (2008) reported that the presence of NaCl improved the retention of biomass in Anammox reactors acting as a granulation promoter. Besides, as it was discussed in Section 7.1, $CaCl_2$ has been used as granulation promoter in anaerobic digesters (Tiwari *et al.*, 2006). The concentration tested in both cases was 5 g L^{-1}, which was supposed not to be significantly inhibitory for the Anammox biomass (Kartal *et al.*, 2006; Dapena-Mora *et al.*, 2007; Fernández *et al.*, 2008).

Table 7.1. Experimental strategy.

Run	Flow rate ($L\ h^{-1}$)	Surface velocity ($m\ h^{-1}$)	Re	Salt concentration ($g\ L^{-1}$)
1	25.2	28.7	188	-
2	8.4	9.6	62.8	-
3	7.3	8.3	54.3	-
4	25.2	28.7	188	5 (NaCl)
5	25.2	28.7	188	5 ($CaCl_2$)

7.4. RESULTS AND DISCUSSION

7.4.1. Calibration

A calibration was performed for each of the experiments in order to obtain the relationship between the amplitude of the signal and the mass of biofilm. Sample collectors were periodically removed during the experiment at different stages of deposit formation and the mass of biofilm was measured in terms of VSS. The obtained calibration equations are the following (Equations 7.2 to 7.6, which correspond to tests 1 to 5, respectively):

$m_b = 182\ A_{norm} + 0.16$ Run 1 (7.2)

$m_b = 397\ A_{norm} - 0.13$ Run 2 (7.3)

$m_b = 207\ A_{norm} - 0.29$ Run 3 (7.4)

$m_b = 1228\ A_{norm} - 1.13$ Run 4 (7.5)

$m_b = 188\ A_{norm} + 4.27$ Run 5 (7.6)

Where m_b is the specific mass of biofilm in each case (g VSS m^{-2}) and A_{norm} is the normalized amplitude of the vibration (dimensionless, calculated respect to the amplitude of the vibration without biofilm in the system).

7.4.2. Effect of the shear stress

In order to study the effect of the shear stress, three experiments, lasting for about 1 month each, were performed. According to Table 7.1, these experiments were performed at different flow rates. The results are presented directly in terms of specific mass of biofilm (Figure 7.3), calculated with the raw data from the MSS and the calibration performed in each case (Section 7.4.1).

The initial trend in the three cases was similar, with a fast adhesion phase lasting for approximately 5 days. However, it was observed that this adhesion phase continued to reach a maximum in the test with the highest shear stress (Re=188). After one week, the specific mass of biofim was about 3 g VSS m^{-2}. Then the shear stress was enough to detach about two thirds of the mass and finally the biofilm reached a stable state with a specific mass value about 1-1.2 g VSS m^{-2}. One hypothetical explanation for this detachment phenomenon was that the start/stop cycles of the positive displacement pump added some extra shear stress. This explanation was based on the observation of the flow cell during the start up phases of the pump. The final stable state of the biofilm would agree

with the reported by Vieira *et al.* (1993) for the properties of biofilms subjected to high shear stress conditions. Besides, Pereira *et al.* (2008) also reported that biofilms formed under high stress are more dense and stable.

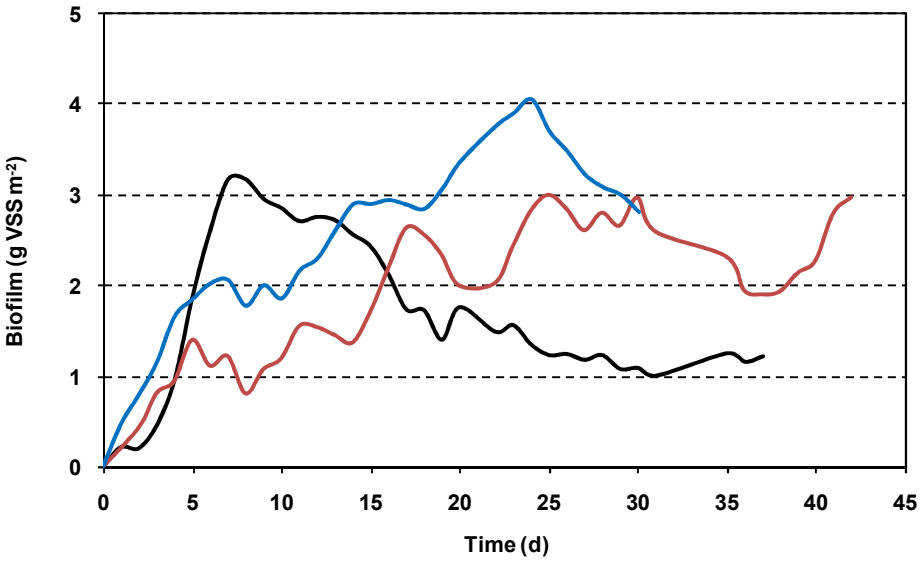

Figure 7.3. Biofilm development during the experiences at different flow rates: Black line, Re=188; red line, Re=62.8; blue line, Re=54.3.

In the case of the two experiences with the lower shear stress (Re=62.8; Re=54.3) the behaviour was similar. After the initial fast adhesion, the development of the biofilm reached a maximum. However, in these cases the biofilm was not as stable as when the shear stress was higher. Therefore, periods of increase and decrease of the specific mass were observed.

These second and third experiences were modelled by using the mathematical equation developed by Melo and Vieira (1999) for the growth of *Pseudomonas fluorescens*:

$$m_b = m_b^{\infty} (1 - e^{-bt}) \qquad (7.7)$$

Where the specific mass of the biofilm at any time (m_b; g VSS m^{-2}) is related to the two parameters of the model: the specific mass of the biofilm at infinite time (m_b^{∞}; g VSS m^{-2}) and the kinetic constant (b; d^{-1}). The values of the model parameters for the second and the third experiences were calculated (Table 7.2). Figure 7.4 presents the comparison of the measured development of the biofilm during tests 2 and 3 and the dependency obtained from the model.

Table 7.2. Values of the model parameters for tests 2 and 3.

Test	m_b^{∞} (g VSS m^{-2})	b (d^{-1})
2	2.66	0.088
3	3.71	0.090

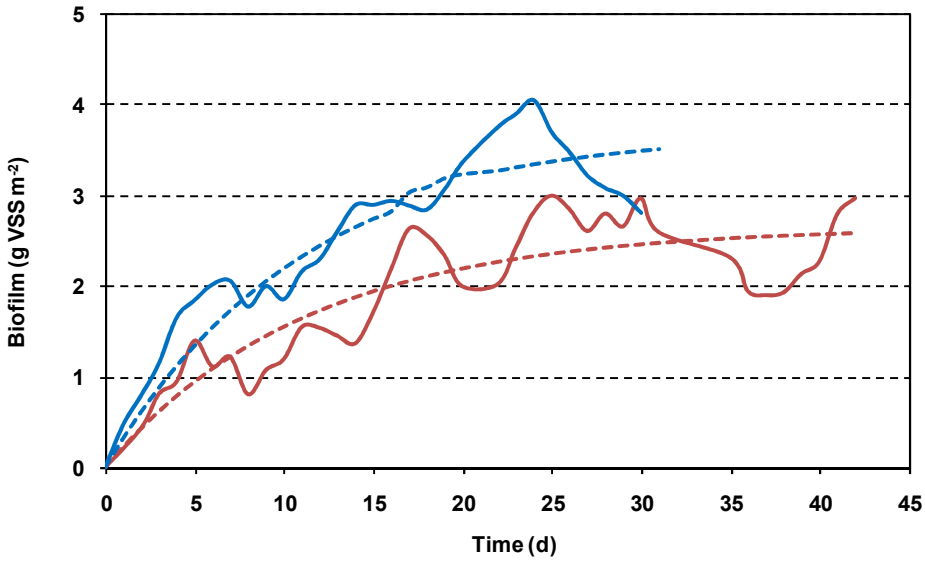

Figure 7.4. Biofilm development (continuous lines) and model (discontinuous lines) for the experiences at Re=62.8 (red) and Re=54.3 (blue).

Despite the model describes well the trend in both experiences, it is important to remember that it was developed for a bacterial specie (*Pseudomonas*) with a doubling time much shorter than Anammox. Therefore, the interpretation of the model is different and in this case it might be representing mainly the adhesion and initial formation of the biofilm, while in the case reported by Melo and Vieira (1999) it may be representing the bacterial growth.

Despite the trend of the biofilm formation during the first experience was different compared to the ones at the lower Re, it is still possible to estimate a value about 1.1 g VSS m^{-2} for the specific mass of the biofilm at infinite time (average of the specific mass of biofilm during the last week of the test). If this value is compared together with the ones included in Table 7.2, the specific mass of biofilm at infinite time was growing with the decrease on the shear stress.

7.4.3. Effect of salinity

Two salts were employed in order to research the effects of salinity on the Anammox biofilm formation, sodium chloride and calcium chloride. When the sodium chloride was tested (Figure 7.5) the behaviour obtained was similar to the observed during test 1, done at the same flow rate (i.e. shear stress). An initial very fast attachment phase was observed, followed by a phase with some instability of the biofilm and partial detachment. Finally the biofilm (Figure 7.6) achieved a relative stability at a specific mass about 6 g VSS m^{-2}. The main difference of this experience compared to test 1 was that both the initial attachment rate and the final specific biofilm mass observed were much higher. This fact can be attributed to the presence of the sodium chloride.

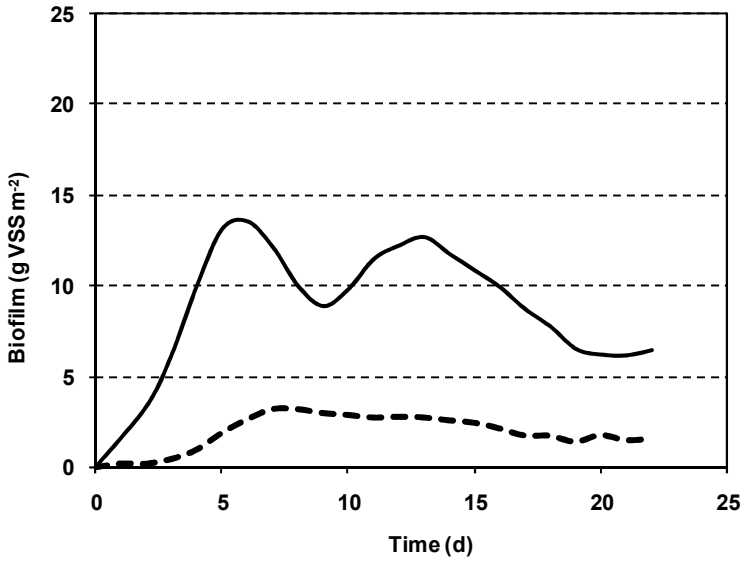

Figure 7.5. Biofilm development during the fourth experience, in presence of 5 g L^{-1} of sodium chloride (continuous line). For comparison, the analogous test at Re=188 without NaCl is displayed with dotted line.

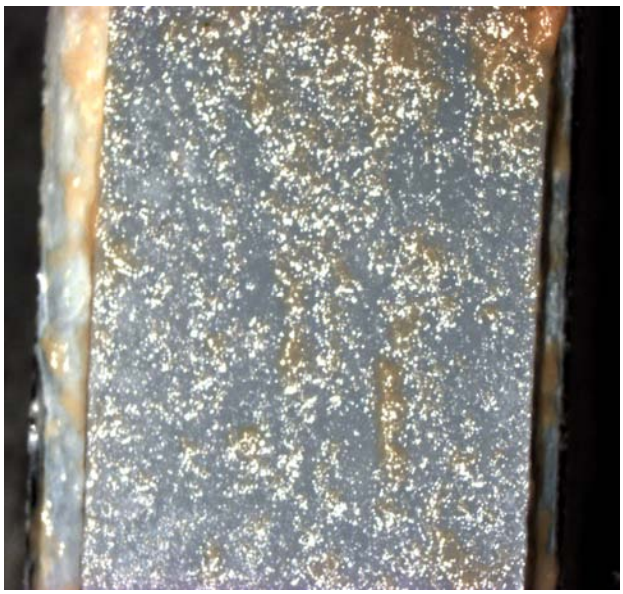

Figure 7.6. Sample of biofilm on the PVC surface of a removable sample collector, 6.5x, day 7 of test 4 (with NaCl).

According to the cation bridging theory proposed by Schmidt and Ahring (1994) and Hulsoff Pol *et al.* (2004) and taking into account that sodium cation is monovalent, it cannot form any bridge between the cell and the support surface. Thus, its effects can be hypothetically explained by other mechanism. It is based on the fact that higher ionic strength of the liquid medium can help to reduce electrostatic double-layer repulsion forces, according to DLVO theory. The ionic strength of the medium before salt addition was about 0.02 M and the increase caused by the addition of NaCl was about 0.09 M. Morisaki and Tabuchi (2009) tested several bacterial species and they reported that the rate of attachment to a surface increased with the ionic strength, which agrees with Figure 7.5. The same behaviour was reported by Poortinga *et al.* (2001). Furthermore, Zhu *et al.* (2009) reported that deposition of Extracellular Polimeric Substances (EPS) increased with increasing ionic strength, which confirmed the predicted by DLVO theory. Besides, they observed that deposition efficiency was higher in divalent solutions than in monovalent solutions. Extracellular polymeric substances (EPS) play an important role in cell aggregation, cell adhesion, and biofilm formation (Dogsa *et al.*, 2005). Thus, deposition of EPS can be the first step of the microbial adhesion when a biofilm is formed. Apart from this phenomenon, some incorporation of the salt into the biofilm was observed and swelling phenomena could take place. In fact the biofilm samples had an average of 37% of inorganics, compared to < 1% for the samples taken during the first three tests when no salt was added.

The behaviour of the biofilm in presence of calcium chloride was similar to the tests 1 and 4 focusing on the first fast attachment phase (Figure 7.7). However, an important difference was observed in this case. After this attachment phase, which reached about 21 g VSS m^{-2}, the biofilm (Figure 7.8) was stable and not a very significant detachment was observed. So, the effect of this salt in stabilizing a thicker biofilm was much more important than the observed with sodium chloride. This can be probably explained by cationic bridging (Schmidt and Ahring, 1994;

Hulsoff Pol *et al.*, 2004) together with the mechanisms previously explained for sodium chloride. The increase of ionic strength of the medium caused by the addition of $CaCl_2$ was about 0.14 M, higher than the caused by NaCl. Furthermore, Sobeck and Higgins (2002) reported that cationic bridging theory is the one which best described the role of Ca^{2+} in bioflocculation. It is important to remark that the incorporation of calcium chloride into the biofilm matrix was happening in a bigger extent than during test 4 and biofilm samples had an average of 53% of inorganics.

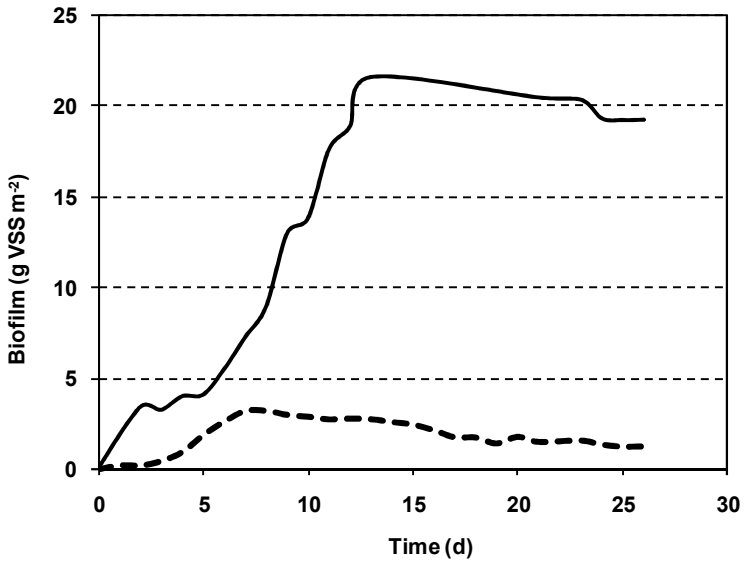

Figure 7.7. Biofilm development in presence of 5 g L^{-1} of calcium chloride continuous line). For comparison, the analogous test at Re=188 without $CaCl_2$ is displayed with dotted line.

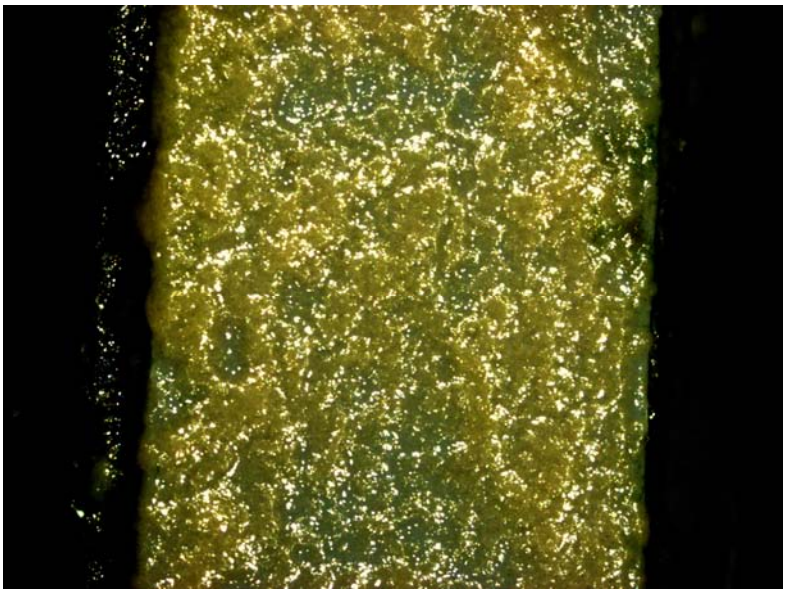

Figure 7.8. Sample of biofilm on the PVC surface of a removable sample collector, 6.5x, day 25 of test 5 (with $CaCl_2$).

The main potential disadvantage of this strategy (high salinity) in order to develop thick Anammox biofilms is that very high salinity was reported as potentially negative for the activity of the biomass (Trigo *et al.*, 2006; Dapena-Mora *et al.*, 2007), so if the salt concentration is too high, the biofilm formed might be thick but with a low activity.

7.5. CONCLUSIONS

Anammox bacteria have a good capacity to attach and to form biofilms. The initial adhesion phase is fast and lasts for 5 to 7 days.

Thicker but less stable biofilms are obtained when operating at low shear stress. When the system is operated at higher shear stress, after about 3 weeks, a very stable biofilm is obtained.

The presence of salts favours the formation of Anammox biofilm because of reduction of electrostatic repulsion forces. The effects of the $CaCl_2$ were

stronger than those caused by NaCl probably because of divalent cationic bridging. Incorporation of inorganics into the biofilm was observed in both cases, but it was more important with $CaCl_2$. This effect can be potentially negative for the activity of the biomass.

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Chapter 8

Biofilm system to improve Anammox biomass retention

ABSTRACT. Appropriate biomass retention in reactors 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 chapter Anammox biofilm formation was promoted to improve biomass retention. Zeolite was chosen as inert support for biofilm formation due to its capacity to adsorb ammonia, which is one of the substrates of the Anammox process.

During the operation of the reactor it was observed that the key factor in order to promote the biofilm growth was to maintain relatively low ammonium concentrations in the liquid bulk. This fact can be a limitation, especially during the start up of the system.

Once the biofilm was established and developed, the biomass wash-out was minimized and the concentration of volatile solids in the effluent was lower than 3 mg VSS L^{-1}. As a consequence the biomass concentration in the reactor increased significantly. Besides, the specific Anammox activity of the biomass was also enhanced increasing from 0.35 up to 0.5 g N_2-N (g VSS d)$^{-1}$.

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Selected results were also presented as: **Fernández I., Trigo C., Vázquez-Padín J.R., Mosquera-Corral A., Figueroa M., Campos J.L. and Méndez R.** Biofilm and granular systems to improve Anammox biomass retention. 7th Specialised Conference on small water and wastewater systems. Mérida, Mexico, March 2006.

8.1. INTRODUCTION

The application of the Anammox process is limited by its long start up periods due to very low growth rates and biomass yields of the involved biomass (Jetten *et al.*, 1997). Minimizing the wash-out of biomass from the reactor by improving its retention becomes critical when biomass with long duplication time (0.003 h^{-1}) is used (Strous *et al.*, 1998). In order to reduce the duration of the start up period and provide better conditions to implant the Anammox process at industrial scale, improvements in biomass retention are necessary. One of the first articles dealing with this problem reported the use of sand particles as inorganic carriers for the biomass (Mulder *et al.*, 1995). Despite the system was successfully treating 1.8 g N $(L\ d)^{-1}$, it turned unstable. System instability was also reported by Strous *et al.* (1997) when they operated a fixed bed formed of glass beads. In this case nitrogen bubbles were trapped in the bed, which led to the reduction of the biomass activity and nitrite build-up.

Some more recent technologies successfully employed in order to improve Anammox biomass retention include the use of Sequencing Batch Reactors (SBR) (Dapena-Mora *et al.*, 2004a), membrane reactors (Trigo *et al.*, 2006), granular systems (Lopez *et al.*, 2008; Tang *et al.*, 2009; Vázquez-Padín *et al.*, 2009) and biofilm attached to plastic Kaldnes carriers (Cema *et al.*, 2006). In reactors where biomass grows in form of biofilm or granules, the formation of compact aggregates increases the settling velocity of the biomass and improves its retention. In those cases the amount of sludge growing in suspension is minimized.

One of the contributing factors to the development of biofilms from suspended sludge is the presence of nuclei or bio-carriers for microbial attachment. The attachment of cells to these particles has been proposed as the initial step for biofilm formation. The second step is the formation of a dense and thick biofilm on the cluster of the inert carriers. To enhance sludge aggregation and attachment the inert materials must have

some properties, such as, a high specific surface area and adequate hydrophobicity (Yu *et al.*, 1999). Natural zeolites, also called mineral zeolites, are aluminium silicates with ionic adsorption and exchange capability because of their structure (Englert and Rubio, 2005). These materials fulfil the requirements to be biofilm support, having ion exchange capacities higher than 2 meq $N\text{-}NH_4^+$ g^{-1} and specific areas about 100-200 m^2 g^{-1}. Besides, zeolite particles have been added to anaerobic digesters in order to act as ammonium buffer avoiding ammonium inhibition of the biomass and improving the stability of the system (Tada *et al.*, 2005). It was also observed that these zeolite particles were helping to promote the formation of anaerobic granules (Hulshoff-Pol, 1989). Furthermore, natural zeolites (as clinoptilolite) are widely used around the world as selective ion exchangers capable of retaining ammonium ions from wastewaters.

Some researchers (Yang, 1997; Lahav and Green, 2000; Lee *et al.*, 2001) have demonstrated that zeolite particles improve the operation of nitrification reactors. This improvement affects both the sedimentation properties of the biomass and the performance of ammonium removal. Lee *et al.* (2001) studied the addition of zeolite particles to a submerged membrane bioreactor. They reported that the nitrifying sludge was attached to the particles, which significantly improved the operation of the membrane. More recently, natural zeolites have also been reported to improve the performance of natural wetlands (Stefanakis *et al.*, 2009) and to be a suitable carrier for phosphate-accumulating bacteria immobilization (Hrenovic *et al.*, 2009).

Considering these facts and the similarities between nitrification and Anammox processes regarding the slow growth of both groups of bacteria, it is proposed that the use of zeolite as carrier material for attached growth of Anammox biomass could improve the start up and operation of an Anammox reactor.

8.2. OBJECTIVES

The objective of the present work was to improve the retention of Anammox biomass in a SBR by promoting the biomass aggregation with natural zeolite particles as carrier material. The effects of the zeolite addition on the specific activity of the biomass and the applicability of this strategy at industrial scale were also studied.

8.3. MATERIALS AND METHODS

8.3.1. Experimental set-up

Experiments were carried out in a SBR with 5L of effective volume (Figure 8.1). Temperature was maintained at 33 ºC by using a thermostatic jacket. The pH value was not controlled and ranged between 7 and 8. The complete mixture inside the reactor was achieved using a mechanical stirrer with rotating speed of 150 rpm. The speed was high enough to maintain the zeolites fluidized but not too high to avoid an excessive shear stress, which could affect the Anammox activity (Arrojo *et al.*, 2006). The control of the pumps and different periods of the operational cycles was performed by a PLC system Siemens model Simatic S7-200 CPU224. The cycle had 6 h distributed in four periods: 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 25%, thus the hydraulic retention time (HRT) was 1 d. Zeolite (10 g L^{-1}) was added as support material. This zeolite was clinoptilolite (ZeoCat, Spain) with a 96% degree of purity, nominal ammonium adsorption capacity between 22.4 and 30.8 mg NH_4^+-N (g zeolite)$^{-1}$ and particle size between 0.5 and 1.0 mm (sieved) (Figure 8.2).

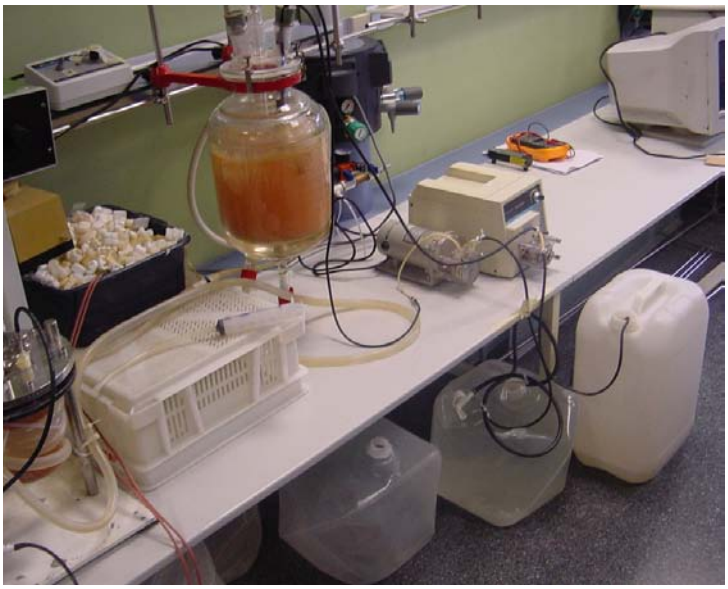

Figure 8.1. Anammox SBR.

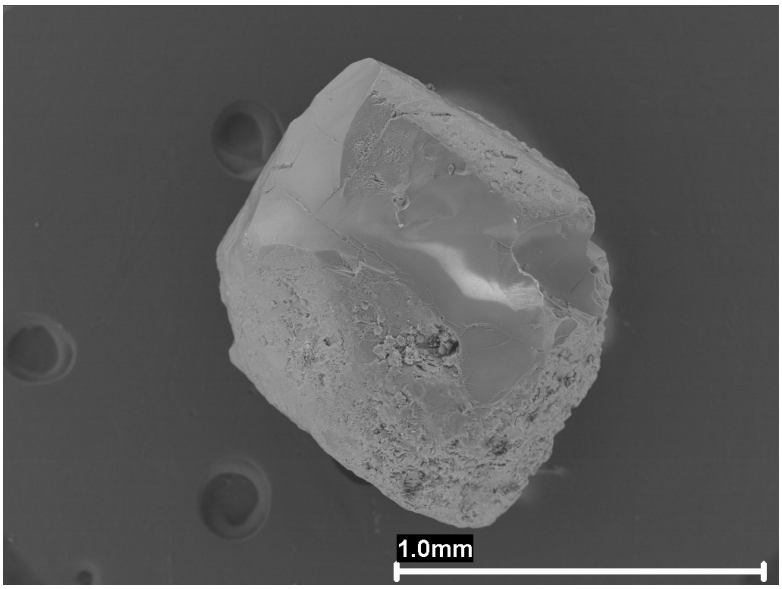

Figure 8.2. Clinoptilolite particle.

8.3.2. Feeding media and operational strategy

The reactor was fed with a synthetic autotrophic medium described in Section 2.4 (Dapena-Mora *et al.*, 2004b). The ammonium to nitrite molar ratio in the feeding media was fixed approximately at 1 to operate in excess of ammonia, except during the last operational period when a NO_2^-–N/NH_4^+–N ratio close to the stoichiometric one (about 1.3 NO_2^-–N/NH_4^+–N according to Strous *et al.*, 1999) was used (Table 8.1) in order to reduce the concentration of ammonium in the reaction medium.

The applied nitrogen loading rate (NLR) was constant at 0.06 g(NH_4^+-N + NO_2^--N) $(L\ d)^{-1}$ during Periods I and II and, in Period III, it was stepwisely increased to 0.60 g(NH_4^+-N + NO_2^--N) $(L\ d)^{-1}$ according to the biomass removal capacity. During Period IV, the NLR was kept constant at 0.43 g(NH_4^+-N + NO_2^--N) $(L\ d)^{-1}$ in order to promote the growth of the biofilm (Section 8.4.3). No biomass from the effluent was returned to the reactor.

Table 8.1. Operational conditions during the different periods.

Period	Time d	Zeolite	$NH_4^+{}_{inf}$ mg N L^{-1}	$NO_2^-{}_{inf}$ mg N L^{-1}	NLR g(NH_4^+–N+NO_2^-–N) $(L\ d)^{-1}$
I	0-13	No	30	30	0.06
II	13-50	Yes	30	30	0.06
III	50-160	Yes	30-300	30-300	0.06-0.60
IV	160-320	Yes	180	250	0.43

8.3.3. Biomass characteristics and concentration measurements

Inoculum. 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 0.24 g VSS L^{-1} with an initial Specific Anammox Activity (SAA) of 0.35 g N $(g\ VSS\ d)^{-1}$.

Biomass measurements. Biomass concentration measurements were performed according to the standard method described in Section 2.2.1

because the zeolite is thermally stable at 550 ºC. Therefore, the mass of zeolite included in the samples was not changed during calcinations and it was accounted as inorganic suspended solids. Consequently, there was no influence of the zeolite on the volatile suspended solids measurement.

8.3.4. Adsorption tests

In order to determine the ammonium adsorption capacity of zeolite (mg NH_4^+–N adsorbed per gram of zeolite) at different concentrations in the liquid phase, adsorption tests were carried out. Temperature was maintained at 33 ºC (thermostatic chamber) and the medium was buffered with a phosphate solution (1.43 g KH_2PO_4 L^{-1} and 7.47 g K_2HPO_4 L^{-1}) to maintain the pH value at 7.8. Zeolite concentration was fixed at 10 g L^{-1} and a mechanical stirrer (120 rpm) was employed (Figure 8.3).

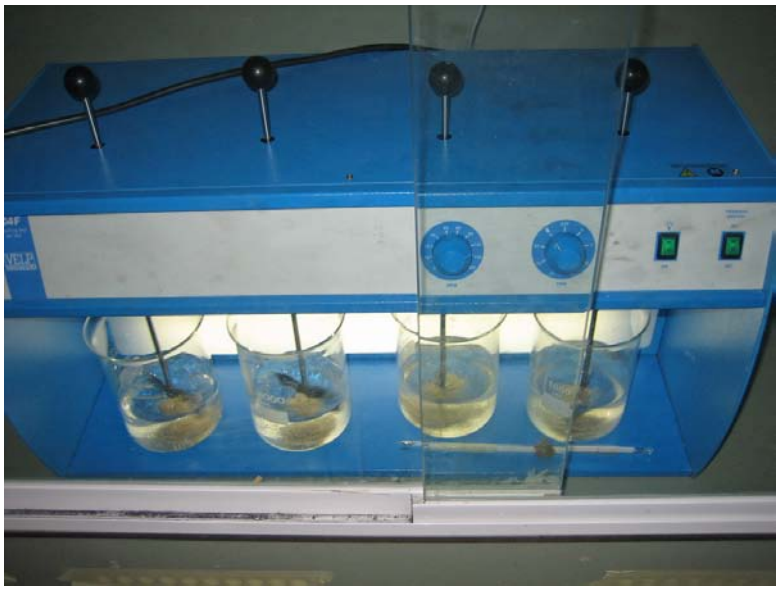

Figure 8.3. Experimental set-up for the adsorption tests.

Four tests were carried out with initial ammonium concentrations of 20, 50, 100 and 500 mg NH_4^+–N L^{-1}. Samples were taken to measure the ammonium concentration in the liquid phase and determine the equilibrium conditions.

8.3.5. Analytical methods

Analytical methods were performed according to Chapter 2.

The monitoring of the biofilm formation and growth was done by taking a representative sample of about 200 particles from the reactor. Then they were distributed in square methacrylate cells with 2 cm of side length. The optimum number of particles in each cell was between 20 and 30. A stereomicroscope Zeiss model Stemi 2000-C was used in order to count the number of support particles covered, uncovered and partially covered.

8.3.6. Specific Anammox activity

To determine the Specific Anammox Activity of the biomass (g N_2-N (g VSS d)$^{-1}$), batch experiments were performed according to the methodology described by Dapena-Mora *et al.* (2007) and in Chapter 2. After SAA tests, biomass concentration measurements were performed as described previously (Section 8.3.3).

8.4. RESULTS AND DISCUSSION

8.4.1. Adsorption tests

Four initial concentrations of ammonium were employed (20, 50, 100 and 500 mg NH_4^+-N L^{-1}) and the observed evolution of the concentration in the liquid phase was as follows (Figures 8.4 and 8.5).

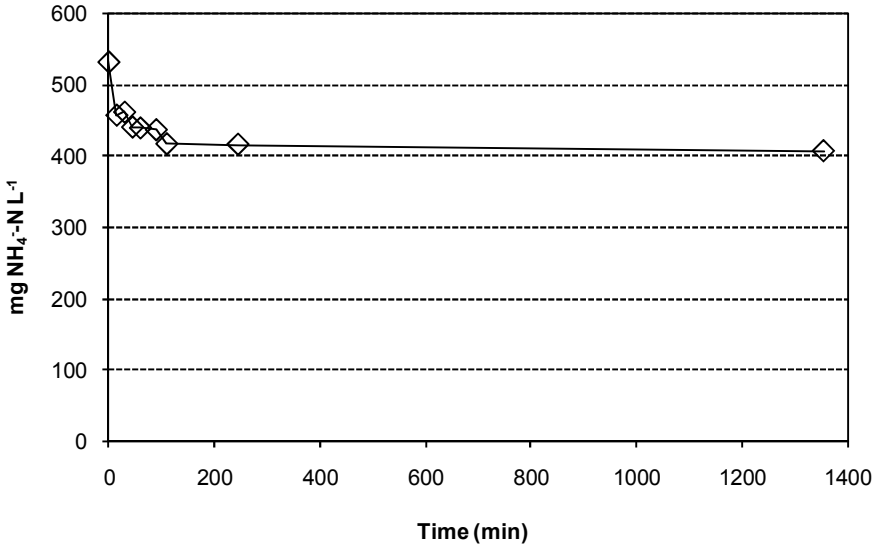

Figure 8.4. Concentration of ammonium in the liquid medium during adsorption test. Initial ammonium concentration: 500 mg NH_4^+-N L^{-1}.

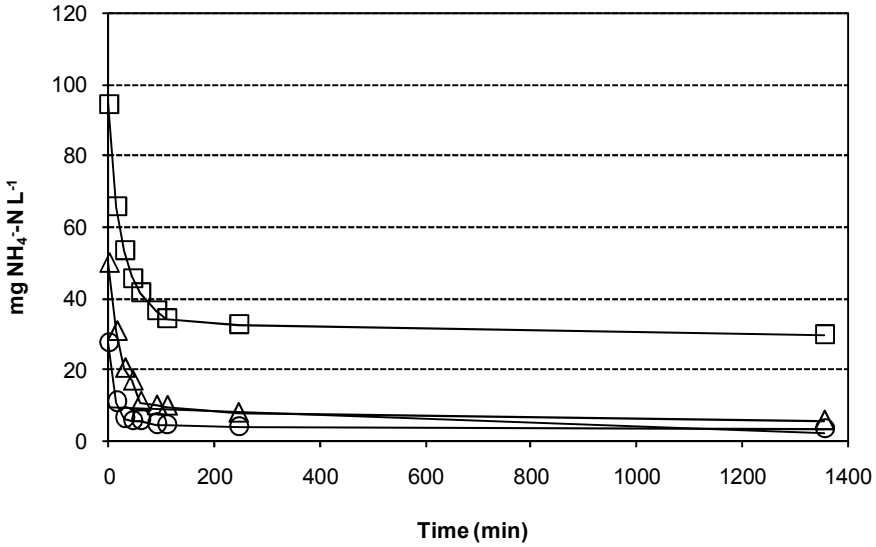

Figure 8.5. Concentration of ammonium in the liquid medium during adsorption tests. Initial ammonium concentrations: 100 mg NH_4^+-N L^{-1} (□); 50 mg NH_4^+-N L^{-1} (Δ); 20 mg NH_4^+-N L^{-1} (○).

The equilibrium data obtained in these tests were subsequently employed in order to fit the Freundlich adsorption model, as it is detailed in Section 8.4.3.

8.4.2. SBR operation

The SBR was initially operated during 13 d (Period I) to obtain stable operational conditions and a complete nitrite removal was observed (Figure 8.6). On day 13, zeolite (10 g L^{-1}) was added to the reactor and the applied NLR was maintained constant at a value of 0.06 g (NH_4^+-N + NO_2^--N) (L d)$^{-1}$ (Period II).

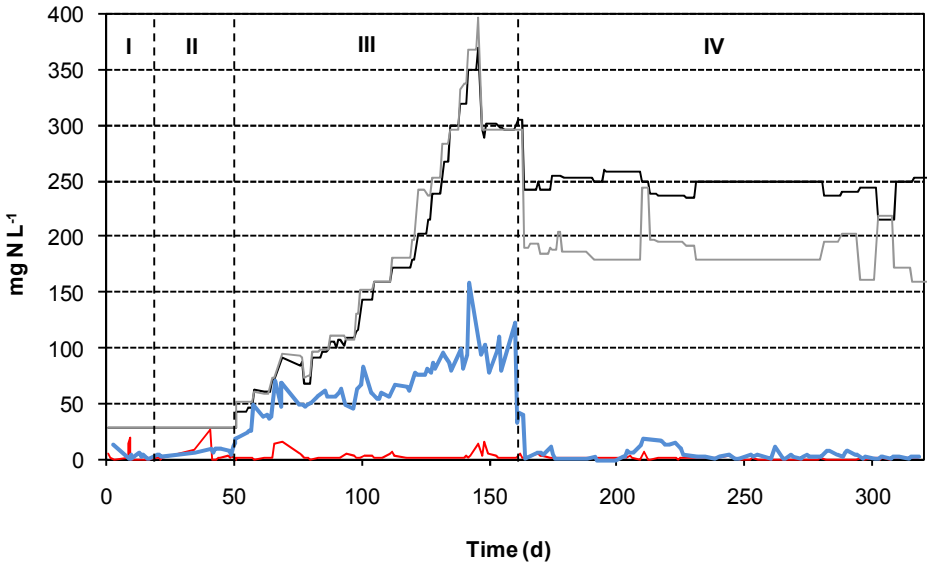

Figure 8.6. Concentrations of nitrogen compounds: ammonium in the influent (■) and effluent (■); nitrite in the influent (■) and effluent (■) (mg N L^{-1}).

From the day 50, the applied NLR was stepwisely increased to 0.6 g (NH_4^+-N + NO_2^--N) (L d)$^{-1}$ by increasing the ammonium and nitrite concentrations in the influent maintaining the equimolar ratio (Period III). The removal efficiency in terms of limiting substrate (nitrite) was higher than 90% during this period. However, since the substrate ratio in the feeding was 1:1, the concentration of ammonium in the effluent was

increasing along the period. Finally, during Period IV, the NLR was decreased to 0.43 g(NH_4^+-N + NO_2^--N) (L d)$^{-1}$ and the substrate ratio was changed to be approximately stiochiometric (about 1.3 NO_2^-–N/NH_4^+–N). Due to this last change, the concentrations of both substrates in the effluent reached very low values and the total nitrogen removal efficiency was higher than 95%.

Dapena-Mora *et al.* (2004c) showed that the SAA determined by batch tests gives useful information about the maximum capacity of the system and it can be used to predict a possible failure of the reactor stability when the NLR applied is increased too much. The Specific Nitrogen Loading Rates (SNLR as g(NH_4^+-N + NO_2^--N) (g VSS d)$^{-1}$) removed by the reactor and the SAA of the biomass along the operational time are shown in Figure 8.7.

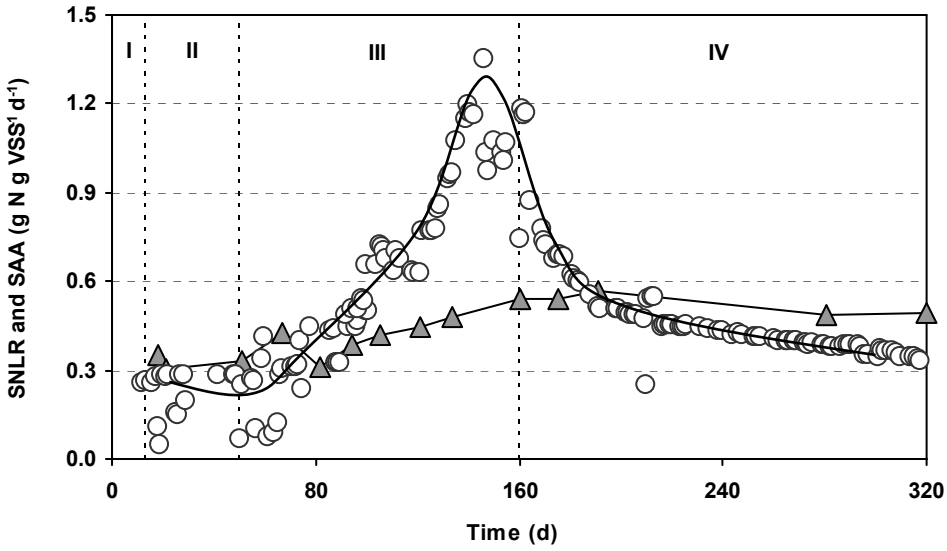

Figure 8.7. SNLR removed (g(NH_4^+-N + NO_2^--N) (g VSS d)$^{-1}$) (○) and SAA of the biomass (g N_2-N (g VSS d)$^{-1}$) (▲).

Contradictory results were obtained during Period III because the efficiency of the reactor was almost 100% with regards to the limiting

substrate (nitrite) but the SAA measured was lower than the SNLR applied (Figure 8.7). These results could be attributed to the difficulty of collection of homogeneous samples due to biomass stratification inside the reactor, which led to not realistic values of biomass concentration and therefore of SNLR. The SAA increased from 0.35 g N_2-N (g VSS d)$^{-1}$ (initial value without zeolite) up to 0.5 g N_2-N (g VSS d)$^{-1}$ (final value with zeolite in the reactor).

8.4.3. Improvement of the biomass retention.

The biomass concentration was maintained constant (around 0.2 g VSS L^{-1}) during Periods I and II but the increase of the applied NLR during Period III caused an increment to 0.63 g VSS L^{-1} (Figure 8.8). In this case biomass retention efficiency close to 90% was estimated. This calculation was done taking into account the consumption of substrates and the productivity of biomass. Loss of biomass by sampling was also considered. Biomass concentration increased up to 1.2 g VSS L^{-1} at the end of Period IV.

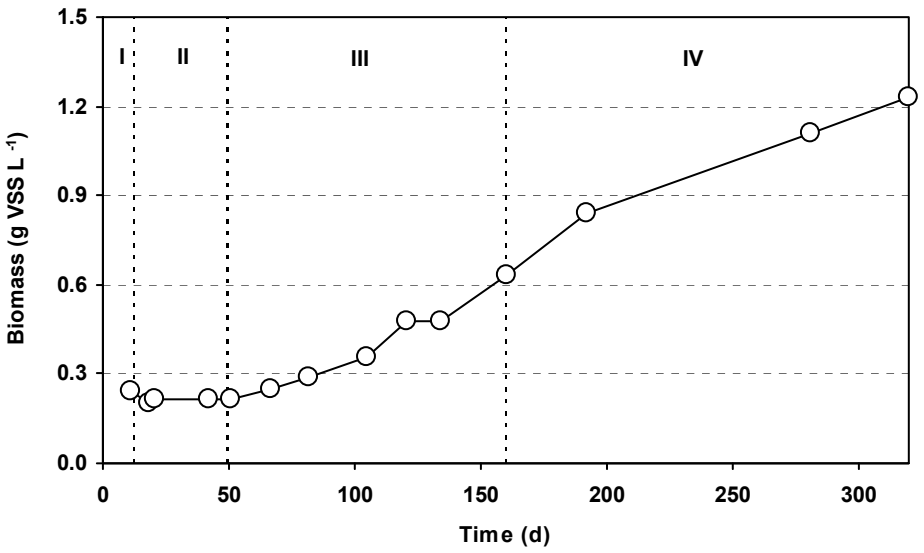

Figure 8.8. Concentration of biomass in the reactor (g VSS L^{-1}) (○).

A quick decrease of the solids concentration in the effluent was observed after the addition of zeolites (Figure 8.9). This caused an increase of the biomass concentration inside the reactor and a consistent increment of the SRT. The very low concentration of solids in the effluent (around 3 mg VSS L^{-1}) during Period IV led to high SRT values (up to 750 d), calculated without taking into account the purge of biomass by sampling.

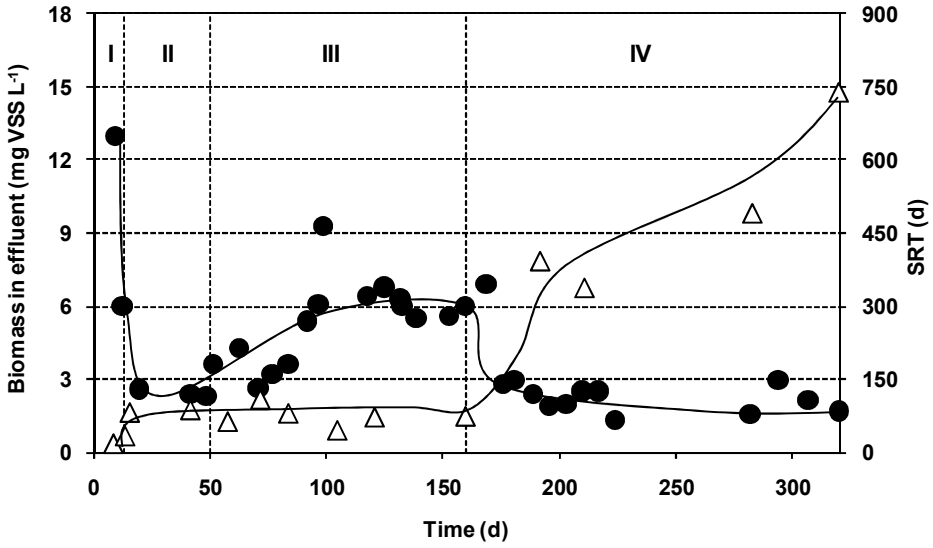

Figure 8.9. Solids concentration in the effluent (mg VSS L^{-1}) (●) and SRT (d) (Δ).

Once the particles of zeolite were added, a biofilm started to growth attached to their surfaces. 30% of fully covered particles and 36% of partially covered particles were observed at the end of Period II (Figure 8.10). Nevertheless, no biofilm formation but the generation of new biomass without zeolite particles as nuclei was observed during Period III.

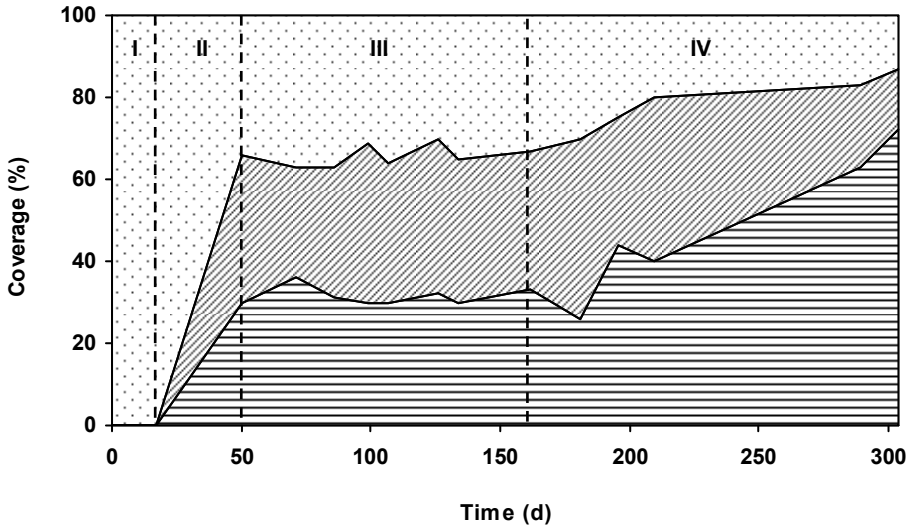

Figure 8.10. Percentages of zeolite fully covered ▤, partially covered ▨ and no covered ⁘.

In order to find the reason why biofilm was formed during Period II but not Period III, it must be taken into account that when both suspended and supported biomass compete for a common substrate present in the liquid phase, suspended biomass has an advantage to access to the substrate due its higher specific surface area. In this work, zeolite was chosen as support since its capacity to adsorb ammonia would favour the access of attached biomass to NH_4^+ and, therefore, it would promote the biofilm formation. Based on this hypothesis, the amounts of ammonia present in the liquid bulk and adsorbed on the particles of zeolite were calculated. Firstly, results obtained from adsorption tests (Section 8.4.1) were fitted to the Freundlich model (Equation 8.1):

$$Qe = 2.06 \times Ce^{0.31} \quad (8.1)$$

Where Qe is the specific amount of ammonium adsorbed (mg NH_4^+–$N_{adsorbed}$ g zeolite^{-1}) and Ce is the concentration of ammonium in the liquid phase (mg NH_4^+-N L^{-1}).

Then, for a fixed value of ammonium concentration in the liquid phase, the amount adsorbed on zeolite (g NH_4^+-N) can be calculated as the product of Qe (g NH_4^+-$N_{adsorbed}$ (g zeolite)$^{-1}$) by the mass of zeolite inside the reactor (g zeolite). The amount of ammonium in the liquid phase is determined as the product of the concentration in the liquid phase (g NH_4^+-N L^{-1}) by the volume of the reactor (L). With these calculations, the percentages of NH_4^+–N available on the zeolite and in the liquid phase were calculated. As it can be seen in Figure 8.11, the most part of the ammonium is adsorbed on zeolite when the concentration in the liquid phase is low but a high ammonium concentration favours its presence in the liquid phase.

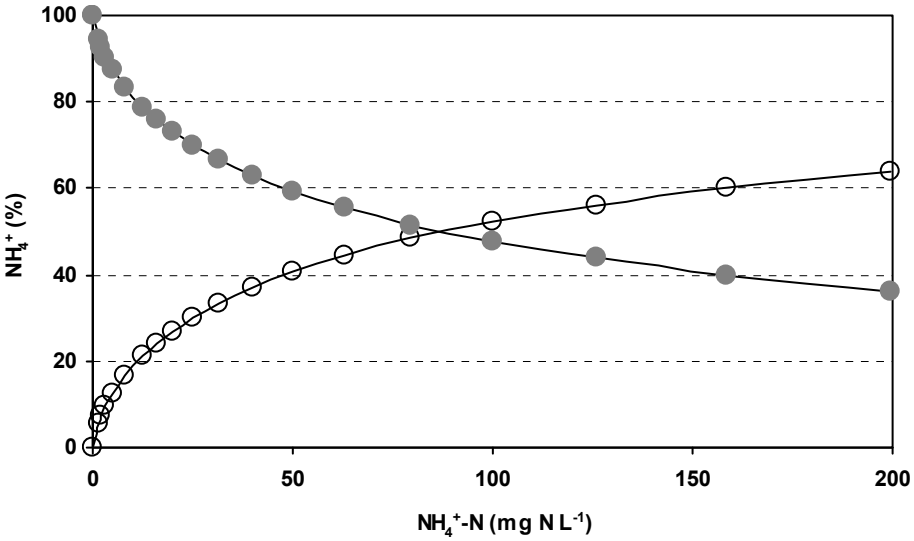

Figure 8.11. Theoretical (predicted) proportions of ammonium adsorbed on the zeolite (●) and present in the liquid phase (○) (%).

Analogous calculations can be used to estimate the amount of ammonium adsorbed and in the liquid medium along the operation of the reactor (Figure 8.12). These estimations are based on the assumption that equilibrium conditions were reached in the reactor, taking into account the length of one operational cycle (6 h) and the adsorption profiles (3 to

4 hours were enough to reach a concentration very near the equilibrium one).

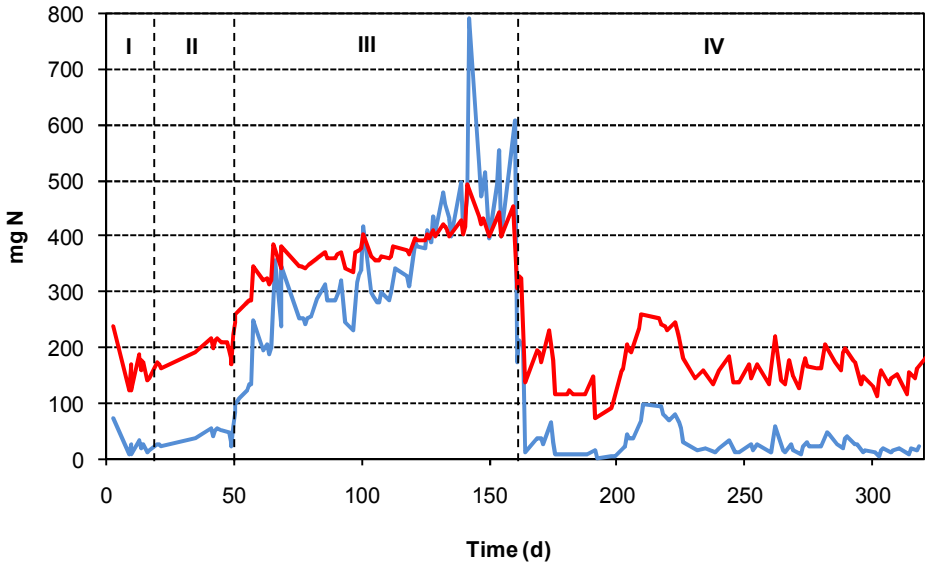

Figure 8.12. Amount of ammonium present in the liquid phase of the reactor (■) and theoretical (predicted) amount of ammonium adsorbed on the zeolite (■) (mg N-NH_4^+).

Initially, the reactor was fed with a synthetic medium which contained a NH_4^+-N/NO_2^--N ratio higher than the stoichiometric one to avoid a possible inhibitory effect of nitrite on the Anammox biomass (Figure 8.6). During Period II, the inlet ammonium concentration was low and, therefore, only a low excess of NH_4^+-N was observed in the reactor (Figure 8.6). However, during Period III, the NLR was increased by raising the inlet concentrations of both nitrite and ammonium. This caused an increment of ammonium in the liquid phase which could be related to the growth of suspended biomass instead of biofilm (Figure 8.10). In order to validate this hypothesis, the reactor was fed with a medium containing a NH_4^+-N/NO_2^--N ratio close to the stoichiometric one (Strous *et al.*, 1999) to reduce the concentration of NH_4^+ in the liquid bulk (Period IV). This strategy led to an

increase of the percentage of the fully covered particles from 30% to around 72% (Figures 8.10 and 8.13).

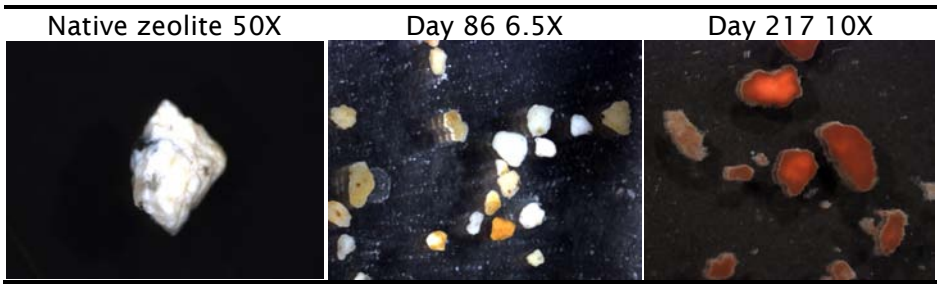

Figure 8.13. Formation of biofilm on zeolite particles during the operational period.

According to Figure 8.12, when the ammonium concentration in the bulk liquid was low (i.e. Periods I, II and IV) the main part of the ammonium was adsorbed on the zeolite, while during Period III, the availability of ammonium in both phases was similar. Therefore, during this phase, the bacteria which were part of the biofilm had no competitive advantage in terms of access to the substrate, compared to the bacteria in the bulk liquid. This fact may be the explanation for the biofilm growth behaviour.

Results show that zeolite particles can be used to enhance the retention of the Anammox biomass. However the high density of these particles implies the necessity of applying a high mixing power into the reactor to keep them in suspension. During the start-up period, such mixing power would promote abrasion produced by bare zeolite particles which can cause detrimental effects on biofilm. Furthermore, the shear stress by itself could cause a decrease on the biomass specific activity (Arrojo *et al.*, 2006). Once the particles are fully covered with biomass this problem is minimized due to the decrease of global particle density and, therefore, lower mixing power is needed.

The growth of biofilm was observed when the ammonium concentration in the bulk liquid was relatively low. This fact implies that one reactor with

this technology has to be fed with a substrate ratio near to the stoichiometric one. In order to achieve this ratio, the previous partial nitrification reactor producing the nitrite has to be carefully controlled. This can be difficult during the start up periods. One additional inconvenient might be the fact that, since Anammox can be inhibited by nitrite (Dapena-Mora *et al.*, 2007), the operation with a significant excess of ammonium would be considered safer, especially if the influent concentrations are not very stable. However, since an amount of ammonium would be adsorbed on the solid support along the operation, the system may act like an "ammonium buffer" and nitrite overloads may be mitigated in some extent. Actually, this can be one of the main advantages of the use of zeolites when compared to other Anammox biofilm technologies (like biofilm on Kaldnes rings; Szatkowska *et al.*, 2007), since zeolites are a kind of "active support" while plastic, glass or other kinds of biofilm supports do not have that ability.

8.5. CONCLUSIONS

From the results obtained in the present work, the addition of zeolite particles as external support material in an Anammox SBR has some advantages. First it allowed a higher enrichment of the Anammox biomass, which was detected by the higher SAA measured. Furthermore, the retention of biomass improved and lower VSS concentrations were measured in the effluent. This fact was correlated to biomass attachment and a reduction of biomass growth in suspension was observed.

However, to promote the growth of biofilms, the concentration of ammonia should be maintained at a low level which can be not so feasible during the start up of the system.

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Conclusiones

A continuación se presentan las conclusiones generales de la presente tesis doctoral, en la cual se evaluó la influencia de diversos parámetros en el proceso Anammox de eliminación autótrofa de nitrógeno, a fin de facilitar su implantación industrial:

1. Efectos de los sustratos

Los sustratos son inhibidores del proceso Anammox tanto a corto como a largo plazo, aunque la capacidad inhibitoria a largo plazo es mayor. Se establecieron unas concentraciones límite de las formas no ionizadas de los sustratos (amonio libre y ácido nitroso), las cuales no es recomendable superar durante la operación de los reactores Anammox. Al tratarse de concentraciones de las formas no ionizadas, son más generalizables y más fáciles de aplicar a diferentes reactores operando a distintos valores de pH y temperatura. Finalmente, los efectos de inhibición a largo plazo son reversibles pero con tiempos de recuperación relativamente largos (alrededor de 1 mes) de ahí que sea importante evitar la desestabilización de los reactores.

2. Efecto de antibióticos de amplio espectro

Las aguas residuales procedentes de la digestión anaerobia de purines son potencialmente muy adecuadas para su post-tratamiento con el proceso Anammox. Sin embargo es esperable que en estas aguas existan concentraciones significativas de antibióticos. Se ha observado que dos de los antibióticos de amplio espectro más comunes (hidrocloruro de tetraciclina y cloranfenicol) son inhibitorios para el proceso Anammox, tanto a corto como a largo plazo. Por lo tanto, se recomienda un

tratamiento específico de degradación (por ejemplo foto-degradación, cloración…) previo al tratamiento con el proceso Anammox.

3. Operación del proceso Anammox a temperaturas relativamente bajas

El proceso Anammox puede ser operado de forma estable y eficiente a temperaturas relativamente bajas (18-20 ºC) a pesar de que su óptimo de temperatura está en el rango 35-40 ºC. Para lograr la operación eficiente a bajas temperaturas una de las claves es el descenso gradual de la temperatura del reactor, que permite un cierto grado de adaptación de la biomasa. La otra clave es contar con una concentración de biomasa relativamente elevada, para compensar el descenso de la actividad Anammox específica debido a la temperatura.

4. Aplicación de superficies de respuesta a la optimización de un sistema Anammox de una etapa (deamonificación)

Los modelos de superficie de respuesta son útiles para conocer la influencia de las diversas variables implicadas en el proceso Anammox. En el caso del sistema de deamonificación evaluado, las variables más importantes fueron la temperatura, el valor de pH y la relación entre amonio libre y ácido nitroso.

La estrategia de control recomendada para reactores de deamonificación, de acuerdo con el modelo, se centra en monitorizar las concentraciones de nitrito, amonio y el valor de pH, calculando así las concentraciones de amonio libre y ácido nitroso. Los valores de estos dos parámetros se podrán controlar directamente mediante el pH o manipulando la velocidad de conversión a través del caudal de aireación.

5. Desarrollo y aplicación de biopelículas Anammox

Las bacterias Anammox tienen una buena capacidad para adherirse a superficies y formar biopelículas. Esto puede emplearse como una ventaja a la hora de mejorar la retención de la biomasa en el interior de los reactores. La adhesión inicial de las bacterias al soporte dura entre 5 y 7 días y se ve favorecida por la presencia en el medio de NaCl o $CaCl_2$. El efecto del $CaCl_2$ es mayor puesto que, además de incrementar la fuerza iónica del medio, es capaz de formar puentes catiónicos divalentes.

El uso de partículas de zeolita natural como soporte en reactores Anammox de biopelícula permite una retención de biomasa muy buena además de un mayor grado de enriquecimiento de dicha biomasa. Para lograr la formación y crecimiento de la biopelícula es necesario que la concentración de amonio en el medio líquido se mantenga baja.

6. Aplicación

La información obtenida en los distintos trabajos presentados en esta tesis es valiosa a la hora de poner en marcha y operar un sistema Anammox a escala piloto o industrial, así como de aplicarlo a aguas residuales diferentes de las que se han tratado hasta ahora. Así se han obtenido varios datos clave para operar el proceso de forma eficiente, mejorar la retención y actividad de la biomasa y establecer un sistema de control del proceso simple y efectivo.

Conclusións

A continuación preséntanse as conclusións xerais da presente tese doutoral, na cal se avaliou a influencia de diversos parámetros no proceso Anammox de eliminación autótrofa de nitróxeno, a fin de facilitar a súa implantación industrial:

1. Efectos dos substratos

Os substratos son inhibidores do proceso Anammox tanto a curto como a longo prazo, aínda que a capacidade inhibitoria a longo prazo é maior. Establecéronse unhas concentracións límite das formas non ionizadas dos substratos (amonio libre e ácido nitroso) que non deben superarse durante a operación dos reactores Anammox. Ó ser concentracións de especies non ionizadas, son máis fáciles de aplicar a diferentes reactores operando en condicións distintas de pH e temperatura. Finalmente, os efectos da inhibición a longo prazo son reversibles pero os tempos de recuperación necesarios son relativamente longos (arredor de 1 mes). Por iso é importante evitar a desestabilización dos reactores.

2. Efecto de antibióticos de amplo espectro

As augas residuais procedentes da dixestión anaerobia de xurro son potencialmente moi adecuadas para o seu tratamento co proceso Anammox. Sen embargo é esperable que nestas augas haxa concentracións significativas de antibióticos. Observouse que dous dos antibióticos de amplo espectro máis comúns (hidrocloruro de tetraciclina e cloranfenicol) son inhibitorios para o proceso Anammox, tanto a curto como a longo prazo. Polo tanto recoméndase un tratamento específico de degradación (por exemplo foto-degradación, cloración…) previo ó tratamento co proceso Anammox.

3. Operación do proceso Anammox a temperaturas relativamente baixas

O proceso Anammox pode ser operado de forma estable e eficiente a temperaturas relativamente baixas (18-20 ºC) a pesar de que o seu óptimo de temperatura está no rango 35-40 ºC. Para lograr a operación eficiente a baixas temperaturas unha das claves é o descenso gradual da temperatura do reactor, o cal permite un certo grao de adaptación da biomasa. A outra clave é ter unha concentración de biomasa relativamente elevada, para compensar o o descenso da actividade Anammox específica debido á temperatura.

4. Aplicación de superficies de resposta á optimización dun sistema Anammox dunha etapa (deamonificación)

Os modelos de superficie de resposta son útiles para coñecer a influencia das distintas variables implicadas no proceso Anammox. No caso do sistema de deamonificación avaliado, as variables máis importantes foron a temperatura, o valor do pH e maila relación entre o amonio libre e o ácido nitroso.

A estratexia de control recomendada para reactores de deamonificación, de acordo co modelo, céntrase en medir as concentracións de nitrito, amonio e o valor do pH, calculando así as concentracións de amonio libre e de ácido nitroso. Os valores destes dous parámetros poderán controlarse directamente mediante o pH ou manipulando a velocidade de conversión a través do caudal de aireación.

5. Desenvolvemento e aplicación de biopelículas Anammox

As bacterias Anammox teñen unha boa capacidade para adherirse ás superficies formando biopelículas. Isto pode empregarse como unha vantaxe á hora de mellorar a retención da biomasa no interior dos

reactores. A adhesión inicial das bacterias ó soporte dura entre 5 e 7 días e é favorecida pola presenza no medio de NaCl ou $CaCl_2$. O efecto do $CaCl_2$ é maior, posto que, ademais de incrementar a forza iónica do medio, é capaz de formar pontes catiónicos divalentes.

O uso de partículas de zeolita natural como soporte en reactores Anammox de biopelícula permite unha retención da biomasa moi boa, ademais dun maior grao de enriquecemento de dita biomasa. Para acadar a formación e crecemento da biopelícula é necesario que a concentración de amonio no medio líquido se manteña baixa.

6. Aplicación

A información obtida nos distintos traballos que se presentan nesta tese é valiosa á hora de poñer en marcha e operar un sistema Anammox a escala piloto ou industrial, así como de aplicalo a augas residuais diferentes das que se veñen tratando ata agora. Así obtivéronse varios datos clave para operar o proceso de forma eficiente, mellorar a retención e a actividade da biomasa e establecer un sistema de control do proceso simple e efectivo.

Conclusions

The main conclusions of this doctoral thesis, which was focused on the evaluation of the influence of several parameters on the Anammox process in order to make easier its industrial implementation, are presented now:

1. Effects of the substrates

The substrates are inhibitors of the Anammox process at short- and long-term exposure, although the long-term effects are more important. Threshold inhibitory concentrations of the unionized species of the substrates were established. It is not advisable to exceed these concentrations in order to avoid inhibition. Since they are concentrations of unionized species, they are easier to be used and applied to different reactors operating at different values of pH and temperature. Finally, long-term inhibition is reversible, but the recovery times are relatively long (about 1 month).

2. Effects of broad-spectrum antibiotics

Supernatant from anaerobic digestion of manure is potentially suitable to be treated by the Anammox process. However, the presence of significant concentrations of antibiotics is expected in these wastewaters. It was observed that two typical broad-spectrum antibiotics (tetracycline hydrochloride and chloramphenicol) are inhibitors of the Anammox process at short- and long-term exposure. Therefore, a specific degradation pre-treatment (e.g. photo-degradation, chlorination...) is advisable when wastewaters containing antibiotics are going to be treated by the Anammox process.

3. Operation of the Anammox process at relatively low temperatures

The Anammox process can be stably and efficiently operated at relatively low temperatures (18-20 ºC) despite its optimum temperature is about 35-40 ºC. One of the key factors in order to achieve the efficient operation at low temperatures is the gradual decrease of the temperature of the reactor. This may result in an adaptation of the biomass. Other key factor is to have a relatively high biomass concentration, which would compensate the decrease of the specific Anammox activity due to the low temperature.

4. Application of response surface models for the optimization of a deammonification (one-step Anammox) system

Response surface models are useful in order to know the influence of different variables on the Anammox process. In the case of the employed deammonification system, the most important variables were the temperature, the value of pH and the ratio between free ammonia and free nitrous acid.

The control strategy recommended for deammonification reactors, according to the model, is focused on monitoring nitrite and ammonium concentrations and the value of pH. Therefore, the concentrations of free ammonia and free nitrous acid can be calculated. These parameters would be directly controlled through the modification of the value of pH or changing the conversion rate by manipulating the aeration flow rate.

5. Development and application of Anammox biofilms

Anammox bacteria have a good adhesion capacity in order to attach on surfaces and form biofilms. This fact is useful to improve biomass retention inside Anammox reactors. The initial adhesion of the bacteria on

the support medium lasts about 5-7 days and it is favoured by the presence of salts (NaCl, $CaCl_2$) in the medium. The effects caused by $CaCl_2$ are stronger because, apart from increasing the ionic strength of the medium, it is able to form divalent cationic bridges.

The use of natural zeolite particles as biomass support in Anammox biofilm reactors allows a better biomass retention in the system. Besides, a better degree of biomass enrichment is achieved. In order to promote the formation and growth of the biofilm it is necessary to have low ammonium concentrations in the reaction medium.

6. Application

The information obtained from the different works presented in this thesis is valuable when a pilot or industrial-size Anammox reactor is going to be started-up or operated. It is also valuable in order to apply the Anammox process to different kinds of wastewaters. Some key data have been obtained which are useful to efficiently operate the process, improve biomass retention and activity and develop a simple and effective control system.

List of Symbols

1. Acronyms

Anammox	Anaerobic Ammonium Oxidation
AOB	Ammonium Oxidizing Bacteria
BABE	Bio Augmentation Batch Enhance
CANON	Completely Autotrophic Nitrogen Removal
CCFC	Central Composite Face Centered
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CSTR	Continuous Stirred Tank Reactor
Cy3	Indocarbocyanine
DAPI	4,6- diamidino-2-phenylindole
DLVO	Deriaguin-Landau-Verwey-Overbeek theory
DNA	Deoxyribonucleic acid
EPS	Extracellular Polimeric Substances
FBR	Fluidized Bed Reactor
FISH	Fluorescent In Situ Hybridization
FLUOS/FITC	5(6)-carboxyfluorescein-N-hydroxysuccinimide ester
MSS	Mechatronic Surface Sensor
NOB	Nitrite Oxidizing Bacteria
OLAND	Oxygen-Limited Autotrophic Nitrification-Denitrification
PBS	Phosphate Buffered Saline solution
PLC	Programmable Logic Controller
PMMA	Poly(methyl methacrylate)
PVC	Polyvinyl chloride
rRNA	Ribosomal Ribonucleic Acid
RSM	Response Surface Methodology
SBR	Sequencing Batch Reactor
Sharon	Single reactor system for High-activity Ammonia Removal over Nitrite
UASB	Upflow Anaerobic Sludge Blanket
UCT Process	University of Cape Town Process

WWTP	Wastewater Treatment Plant

2. Symbols

b	Biofilm kinetic constant	d^{-1}
BOD_5	Biodegradable Oxygen Demand at 5 days	mg O_2 L^{-1}
Ce	Concentration of ammonium in the liquid phase (adsorption equilibrium)	mg NH_4^+-N L^{-1}
COD	Chemical Oxygen Demand	mg O_2 L^{-1}
ENLR	Effective Nitrogen Loading Rate	g N $(L\ d)^{-1}$
FA	Free Ammonia	mg N L^{-1}
FNA	Free Nitrous Acid	µg N L^{-1}
HRT	Hydraulic Retention Time	d
IC50	50% Inhibitory Concentration	mg L^{-1}/µg L^{-1}
K_O	Oxygen affinity constant	mg O_2 L^{-1}
m_b	Specific mass of biofilm	g VSS m^{-2}
N	Number of individual test	—
NLR	Nitrogen Loading Rate	g N $(L\ d)^{-1}$
p. e.	Population Equivalent	—
Qe	Specific amount of ammonium adsorbed (adsorption equilibrium)	mg NH_4^+–$N_{adsorbed}$ g $zeolite^{-1}$
Re	Reynolds number	—
SAA	Specific Anammox Activity	g N $(g\ VSS\ d)^{-1}$
	(Related to biofilm area)	g N $(m^2\ d)^{-1}$
SRT	Solids/Sludge Retention Time	d
SVI	Sludge Volume Index	mL $(g\ VSS)^{-1}$
T	Temperature	ºC
TKN	Total Kjeldahl Nitrogen	g TKN-N L^{-1}
TN	Total Nitrogen concentration	mg (NH_4^+-N+ NO_2^--N) L^{-1}
TSS	Total Suspended Solids	mg L^{-1}
VSS	Volatile Suspended Solids	g L^{-1}

List of Publications

1. International Journal Publications

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

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6. Contributions to Conferences

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This doctoral thesis is focused on biological treatment of wastewater and, specifically, on nitrogen removal. The use of technologies based on anaerobic ammonium oxidation (Anammox) is a relatively new alternative to treat wastewaters with low COD/N ratios. This process is carried out by autotrophic bacteria which, in anoxic conditions, combine ammonium and nitrite into nitrogen gas and a small amount of nitrate. It has many advantages compared to the traditional nitrification/denitrification process but there are still some important research issues which should be addressed to increase the chances to apply the process at full scale. Some of these issues are the focus of the present doctoral thesis: the improvement of the biomass retention in the reaction systems, the evaluation of the effects of the potential inhibitors and the application of the process at the mesophilic range of temperature.

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