UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Ingeniería Química

Autotrophic denitrification for treatment of wastewater with high concentration of sulphur and nitrogen compounds

Memoria presentada por
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Informan:

Que la memoria titulada "Autotrophic denitrification for treatment of wastewater with high concentration of sulphur and nitrogen compounds", que para optar al grado de Doctor de Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Doña María del Carmen Fajardo Ortíz, 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, 26 de Enero de 2011.

Ramón Méndez Pampín	Anuska Mosquera Corral	José Luis Campos Gómez

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Table of Contents

Objectives and Summary

Anthropogenic activities have contributed to the imbalance of nitrogen and sulphur natural cycles which causes many negative effects in nature due to the emissions of sulphur and nitrogen compounds and their transformations, e.g. rain acid, eutrophication, bad odours etc.

To avoid such negative effects on environment, effluents containing high concentrations of both nitrogen and sulphur compounds must be treated previously to their discharge. Nitrification/denitrification is the conventional process to remove nitrogen compounds from wastewater. However, when effluents are characterized by low C/N ratios, the nitrogen removal efficiency is limited by the low amount of organic matter available to carry out the denitrification process. In this aspect, autotrophic denitrification supposes a suitable alternative to heterotrophic denitrification. This process is carried out by Thiobacillus-type bacteria which combine nitrate and reduced sulphur compounds to generate dinitrogen gas and sulphate or elemental sulphur. Therefore, the autotrophic denitrification process is able to simultaneously remove both nitrogen and sulphur compounds without presence of organic matter.

Compared to other processes, this treatment would also permit to minimize the environment impact in several aspects: the protection of ozone layer by decreasing the nitrogen oxides emission, the decrease of the greenhouse effect by reducing the energy needed for the process, the reduction in the raw materials (as organic matter) consumption and reduction of the amount of sludge generated due to its low sludge production.

The present thesis is focused on studying the autotrophic denitrification process. The main objective of this project was the operation of a Sequencing Batch Reactor (SBR) under autotrophic denitrifying conditions for simultaneous removal of nitrate and sulphide, being the general objectives:

- Optimize the enrichment of autotrophic denitrifying biomass to be used as seed for the SBR system.
- Test the operation of an autotrophic denitrifying sequencing batch reactor for simultaneous removal of nitrate and sulphide.
- Test the operation of a nitrifying/autotrophic denitrifying system for the post-treatment of an effluent coming from an anaerobic digester treating fish canning industry wastewater.

- Study the influence of different parameters on autotrophic denitrification when sulphide is used as electron donor.

- Characterize, by means of molecular techniques, the evolution of the microbial community during its enrichment and along the operational period of the SBR system.

These objectives have been developed in the different chapters of the thesis in the following way:

Chapter 1. It consists in a bibliographic review of the autotrophic denitrification process, including aspects such as the stoichiometric, kinetic and molecular aspects and biochemistry of the process. The effects of different factors (temperature, pH, inhibitors, S/N ratio, etc.) and the description of the different microorganisms able to carry out the autotrophic denitrification process are also included. Finally the feasibility of applying this process is presented.

Chapter 2. The analytical methods used in this work are described. It comprises the conventional parameters used for wastewater (nitrogen compounds, pH and solids) and biomass (sludge volumetric index, specific activity) characterization. The molecular techniques used to identify the different populations present in the biomass are also described: FISH, DGGE, PCR and sequencing.

Chapter 3. In this chapter, autotrophic denitrifying biomass was enriched from sludge collected at an anaerobic digester. The initial specific activity of the biomass was 9.5 mg $S_2O_3^{2-}$-S (g SSV)$^{-1}$ d^{-1}. A batch reactor was selected as system to enrich this kind of biomass due to its high biomass retention capacity.

After the successive feedings of a synthetic medium containing both thiosulphate and nitrate, autotrophic denitrifying biomass was enriched and, on day 95, its specific activity was 187 mg $S_2O_3^{2-}$-S (g SSV)$^{-1}$ d^{-1}.

At the end of the experiment, the capacity of the biomass to use different sulphur compounds was tested. The specific activities obtained were 211, 153 and 11.5 mg NO_3^--N (g SSV)$^{-1}$ d^{-1} using thiosulphate, sulphide and elemental sulphur, respectively.

Chapter 4. A SBR reactor was operated under autotrophic denitrifying conditions during 220 days at Sulphide Loading Rates (SLR) ranging from 200 to 450 mg $S^{2-} \cdot L^{-1} \cdot d^{-1}$.

The removal efficiencies obtained for sulphide and nitrate were 100% and 67%, respectively. The performance of the reactor was greatly influenced by pH. pH values higher than 9.0 caused that sulphide was only oxidized into elemental sulphur which was accumulated inside the reactor. Such pH values also decreased the specific activity of biomass to 10% of its initial value. Only when pH was controlled at a value around 8, the system recovered its capacity to full oxidize sulphide into sulphate.

Chapter 5. A combination of a nitrifying reactor and an autotrophic denitrifying reactor was used for the post-treatment of effluent from an anaerobic digester treating wastewater from fish canning industry. The nitrifying reactor was able to oxidize an Ammonium Loading Rate (ALR) of 314 mg NH_4^+-N·L^{-1}·d^{-1} into nitrate with an efficiency of 100%. This effluent was fed to the autotrophic denitrifying reactor which treated a maximum SLR of 200 mg S^{2-}·L^{-1}·d^{-1}, with an efficiency of 100%. Nevertheless only 30% of nitrate fed was removed due to sulphide limitation and, therefore, the addition of elemental sulphur was proposed as an alternative to improve the nitrate removal efficiency.

The estimated cost of operation of this system, 0.80 €/Kg $N_{removed}$, is lower than that calculated for conventional nitrification/denitrification processes but the application of SHARON/anammox processes is the cheapest option to treat effluents with low C/N ratios. However the combination of nitrification and autotrophic denitrification (using elemental sulphur) processes would present a better operational stability compared to the SHARON/anammox system.

Chapter 6. In this chapter the kinetic aspects of the autotrophic denitrification process were studied.

The autotrophic denitrifying biomass was inhibited at nitrate concentrations over 200 mg NO_3^--N·L^{-1}, the Haldane´s model was the model that best explained this inhibition. The following parameters were determined: V_{max}= 0.470 ± 0.0013 mg N_2·L^{-1}·min^{-1}, Ks= 13.51 ± 0.113 mg NO_3^--N·L^{-1} and K*i*= 355 ± 4.38 mg L^{-1}. Inhibition by nitrite was also observed and it occurred at concentrations four times lower than those found for nitrate. The value of IC_{50} (concentration causing 50% inhibition) was found to be 48 mg NO_2^--N L^{-1}.

The optimum values of temperature and pH to carry out the complete autotrophic denitrification were 35 ºC and 8.0, respectively.

Ammonium and phosphate did not cause inhibitory effects on denitrification at the different concentrations tested. More research would be necessary about the effects of these two compounds.

Chapter 7. In this chapter the microbial community of the autotrophic denitrifying biomass was analyzed by molecular techniques (DGEE, PCR, FISH and sequencing). The results obtained by the FISH technique did not reveal the presence of autotrophic denitrifying biomass in the sludge used as seed, which could explain the low initial specific activity of the biomass. However samples of the enriched biomass showed the predominance of *Thiobacillus denitrificans*. This type of bacteria is greatly influenced by changes in pH which agreed with the low efficiency of the system when the pH values were close to 9. No effects on bacterial population distribution were observed when the synthetic feeding medium was switched by a nitrified effluent of an anaerobic digester treating wastewater from fish canning industry.

Phylogenetic analysis shows the bacterial community was closely related to *Thiobacillus denitrificans* and *Bacteroidetes*. Other two microorganisms able to use some sulphur compounds, *Chlorobi* and a candidate of division JS1, and a semi-anaerobic micro-organism able to use nitrate, closely related to *Stenotrophomonas*, were also detected.

Objetivos y Resumen

Las diversas actividades antropogénicas han contribuido a un desbalance en los ciclos naturales del nitrógeno y azufre, lo que causa muchos efectos negativos en la naturaleza debido a las emisiones de compuestos de nitrógeno y azufre y a sus transformaciones, como por ejemplo, lluvia ácida, eutrofización, malos olores, etc.

Para evitar esos efectos negativos en el medio, los efluentes con altas concentraciones de compuestos de nitrógeno y azufre han de ser tratados antes de su descarga. La nitrificación/desnitrificación es el proceso convencional para la eliminación de nitrógeno de las aguas residuales. Sin embargo, en aquellos casos en que las aguas residuales contienen unas bajas relaciones C/N, la eficacia de eliminación de nitrógeno se encuentra limitada por la baja cantidad de materia orgánica disponible para llevar a cabo la desnitrificación. De esta forma, la desnitrificación autótrofa puede ser una buena alternativa a la desnitrificación heterótrofa. Este proceso lo llevan a cabo bacterias del tipo *Thiobacillus* que utilizan nitrato y compuestos reducidos de azufre para producir nitrógeno gas y sulfato o azufre elemental. Por lo tanto, el proceso de desnitrificación autótrofa es capaz de eliminar simultáneamente compuestos de nitrógeno y azufre sin presencia de materia orgánica.

Comparado con otros procesos, este tratamiento permitiría reducir el impacto al ambiente en varios aspectos como: la protección de la capa de ozono por disminución de emisión de óxidos de nitrógeno, la disminución del efecto invernadero por el menor consumo energético, la reducción del consumo de reactivos (materia orgánica) y de la cantidad de lodos generada debido a la baja producción de biomasa.

La presente tesis se centra en el estudio del proceso de desnitrificación autótrofa. Siendo el objetivo principal de esta tesis la operación de un reactor secuencial (Sequencing Batch Reactor, SBR) en condiciones de desnitrificación autótrofa para la eliminación simultánea de nitrato y sulfuro. Los objetivos generales son:

- Optimización del enriquecimiento de biomasa desnitrificante autótrofa para ser usada como inóculo de un reactor SBR.

- Operación de un reactor SBR desnitrificante autotrófo para la eliminación simultánea de nitrato y sulfuro.

- Operación de un sistema combinado de nitrificación/desnitrificación autótrofa para el tratamiento del efluente generado en un digestor anaerobio tratando las aguas residuales de una industria procesadora de pescado.

- Estudio del efecto de distintos parámetros sobre la desnitrificación autótrofa usando sulfuro como donador de electrones.

- Caracterización de las comunidades microbianas durante la etapa de enriquecimiento y operación de los reactores desnitrificantes autótrofos por medio de técnicas moleculares.

Estos objetivos fueron desarrollados en los distintos capítulos que forman esta tesis de la siguiente forma:

Capítulo 1. En este capítulo se hace una revisión bibliográfica acerca del proceso de desnitrificación autótrofa, incluyendo aspectos como son su estequiometría, parámetros cinéticos y moleculares, así como la bioquímica del proceso. También se incluyen los efectos de diferentes factores (temperatura, pH, inhibidores, relación S/N, etc.) y la descripción de los diferentes microorganismos capaces de llevar a cabo el proceso de desnitrificación autótrofa. Finalmente se presenta la factibilidad de aplicación de este proceso.

Capítulo 2. En este capítulo se describen los métodos analíticos utilizados en esta tesis. Se incluyen los métodos fisicoquímicos comúnmente usados para el análisis de aguas residuales (análisis de compuestos nitrogenados, pH, sólidos, etc.) y los métodos empleados para caracterizar la biomasa (índice volumétrico de lodos y actividades específicas). La evolución e identificación de las diferentes poblaciones microbianas presentes en la biomasa fueron estudiadas por medio de técnicas moleculares como: FISH, DGGE, PCR y secuenciación.

Capítulo 3. En este capítulo se llevó a cabo el enriquecimiento de biomasa desnitrificante autótrofa, para su posterior uso como inóculo de los reactores. La actividad inicial fue de 9,5 mg $S_2O_3^{2-}$-S (g SSV)$^{-1}$ d^{-1}. Con la finalidad de mantener una alta retención de biomasa, se seleccionó un sistema de reacción discontinuo para llevar a cabo el enriquecimiento de la biomasa desnitrificante autótrofa.

Después de una serie de alimentaciones sucesivas con un medio sintético con tiosulfato y nitrato, se logró el enriquecimiento de biomasa desnitrificante autótrofa. A los 95 días su actividad específica fue 187 mg $S_2O_3^{2-}$-S (g SSV)$^{-1}$ d^{-1}.

Al final del experimento, la biomasa obtenida fue capaz de utilizar diferentes compuestos azufrados como donadores de electrones para llevar a cabo el proceso de desnitrificación autótrofa. Las actividades específicas obtenidas fueron 211, 153 y 11,5 mg NO_3^--N (g SSV)$^{-1}$ d^{-1} empleando tiosulfato, sulfuro y azufre elemental, respectivamente.

Capítulo 4. En este capítulo se llevo a cabo la desnitrificación autótrofa en un reactor SBR. El reactor fue operado durante 220 días, con una velocidad de carga de sulfuro (SLR) de 200 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$ hasta 450 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$. Las eficacias de eliminación de sulfuro y nitrato fueron de 100% y 67%, respectivamente. La eficacia del reactor fue seriamente afectada por el valor de pH. Valores superiores a 9,0 causaron que el sulfuro fuera oxidado sólo a azufre elemental, que se acumuló en el interior del reactor. Dichos valores de pH también causaron un descenso del 10% en la actividad específica de la biomasa. Sólo cuando el pH se mantuvo controlado en un valor alrededor de 8 se logró la recuperación de la capacidad del sistema para oxidar completamente el sulfuro a sulfato.

Capítulo 5. En este capítulo un sistema combinado de nitrificación/desnitrificación autótrofa fue usado para el postratamiento de un efluente proveniente de un digestor anaerobio, el cual trataba las aguas residuales generadas en una industria procesadora de pescado. El reactor nitrificante fue capaz de oxidar a nitrato una velocidad de carga nitrogenada (ALR) de 314 mg NH_4^+-N$\cdot L^{-1}\cdot d^{-1}$ con una eficacia del 100%. El efluente de este reactor fue alimentado a un reactor desnitrificante autótrofo que trataba una máxima carga de sulfuro aplicada de 200 mg S^{2-} L^{-1} d^{-1}, con una eficacia del 100%. Sin embargo sólo se eliminó el 30% del nitrato alimentado debido a limitación por sulfuro. Por lo tanto la adición de azufre elemental se propone como una alternativa para mejorar la eliminación de nitrato en este sistema.

El coste de operación estimado para este sistema es de 0,80 €/Kg $N_{eliminado}$, que es inferior al calculado para el sistema convencional de nitrificación/desnitrificación. Sin embargo el sistema SHARON/anammox es el proceso más económico para tratar efluentes con bajas relaciones C/N. Una ventaja del sistema combinado de nitrificación y desnitrificación autótrofa (usando azufre elemental) podría ser su mayor estabilidad frente a la del proceso SHARON/anammox.

Capítulo 6. En este capítulo se estudiaron los aspectos cinéticos del proceso de desnitrificación autótrofa.

Los resultados obtenidos a diferentes concentraciones de sulfuro/nitrato muestran que el nitrato tiene un efecto inhibitorio sobre la desnitrificación a concentraciones superiores a 200 mg NO_3^--N·L^{-1}. El modelo de Haldane fue usado para explicar este comportamiento, obteniéndose los siguientes parámetros cinéticos: V_{max}= 0,470 ± 0,0013 mg N_2·L^{-1}·min^{-1}, K*s*= 13,51 ± 0,113 mg NO_3^--N·L^{-1} y K*i*= 355 ± 4,38 mg L^{-1}. También se observó inhibición por nitrito y ésta ocurrió a concentraciones cuatro veces inferiores a las encontradas para el nitrato. El valor de IC_{50} (concentración que produce el 50% de inhibición) obtenido fue de 48 mg NO_2^--N L^{-1}.

Los valores óptimos encontrados de temperatura y pH para llevar a cabo la desnitrificación autótrofa fueron 35 ºC y 8,0 respectivamente.

No se observaron efectos inhibitorios causados por amonio, amonio libre y fosfato a las distintas concentraciones empleadas. Son necesarios más estudios acerca de los efectos de estos compuestos.

Capítulo 7. En este capítulo se analizaron las comunidades microbianas de la biomasa desnitrificante autótrofa por medio de técnicas moleculares (FISH, PCR, DGGE y secuenciación). Los resultados obtenidos mediante la aplicación de la técnica FISH revelaron que el lodo utilizado como inóculo carecía de bacterias desnitrificantes autotróficas, lo cual explica la baja actividad desnitrificante al inicio de este trabajo. Sin embargo la biomasa enriquecida y la biomasa presente en los reactores operados en esta tesis estuvieron caracterizadas por una alta densidad de bacterias identificadas como *Thiobacillus denitrificans*. Esta población es muy sensible a los cambios de pH, lo cual explica la baja eficacia del sistema cuando el pH alcanzó valores alrededor de 9. No se observó un cambio en la población cuando el medio de alimentación sintético fue remplazado por el efluente nitrificado de un digestor anaerobio que trataba aguas procedentes de la industria conservera.

El análisis filogenético mostró que la comunidad microbiana que conforma el lodo está caracterizada por bacterias del tipo *Thiobacillus denitrificans* y *Bacteroidetes* principalmente, además de dos grupos de bacterias capaces de usar alguna fuente azufrada, *Chlorobi* y un candidato de la división JS1, y un grupo de bacterias semi-anaerobias capaces de utilizar nitrato relacionadas con *Stenotrophomonas.*

Obxectivos e Resumo

As diversas actividades humanas contribuíron á perda do balance nos ciclos naturais do nitróxeno e xofre, o que causa moitos efectos negativos na natureza debido ás emisións de compostos de nitróxeno e xofre a ás súas transformacións, como por exemplo, chuvia ácida, eutrofización, malos olores, etc.

Para evitar estes efectos negativos no medio, os efluentes con elevadas concentracións de compostos de nitróxeno e xofre deben ser tratados antes da súa descarga. A nitrificación/desnitrificación é o proceso convencional para a eliminación de nitróxeno das augas residuais. Sen embargo, naqueles casos nos que as augas residuais conteñen unhas baixas relacións C/N, a eficacia da eliminación de nitróxeno está limitada pola baixa cantidade de materia orgánica dispoñible para levar a cabo a desnitrificación. Desta maneira, a desnitrificación autótrofa pode ser unha boa alternativa á desnitrificación heterótrofa. Este proceso lévano a cabo bacterias do tipo *Thiobacillus* que empregan nitrato e compostos reducidos de xofre para producir nitróxeno gas e sulfato ou xofre elemental. Polo tanto, o proceso de desnitrificación autótrofa é capaz de eliminar simultaneamente compostos de nitróxeno e xofre sen presenza de materia orgánica.

Comparado con outros procesos, este tratamento permitiría reducir o impacto ó ambiente en varios aspectos como: a protección da capa de ozono pola diminución de emisión de óxidos de nitróxeno, a diminución do efecto invernadoiro polo menor consumo de enerxía, a redución do consumo de reactivos (materia orgánica) e da cantidade de lamas xerada debido á baixa produción de biomasa.

A presente tese céntrase no estudo do proceso de desnitrificación autótrofa. O seu obxectivo principal é a operación dun reactor secuencial (Sequencing Batch Reactor, SBR) en condicións de desnitrificación autótrofa para a eliminación simultánea de nitrato e sulfuro. Os obxectivos xerais son:

- Optimización do enriquecemento de biomasa desnitrificante autótrofa para o seu uso como inóculo dun reactor SBR.
- Operación dun reactor SBR desnitrificante autótrofo para a eliminación simultánea de nitrato e sulfuro.
- Operación dun sistema combinado de nitrificación/desnitrificación autótrofa para o tratamento do efluente xerado nun dixestor anaerobio tratando as augas residuais dunha industria procesadora de peixe.

- Estudo do efecto de distintos parámetros na desnitrificación autótrofa empregando sulfuro como doador de electróns.

- Caracterización das comunidades microbianas durante a etapa de enriquecemento e operación dos reactores desnitrificantes autótrofos por medio de técnicas moleculares.

Estes obxectivos foron desenvolvidos nos diferentes capítulos que forman esta tese da seguinte maneira:

Capítulo 1. Neste capítulo faise unha revisión bibliográfica do proceso de desnitrificación autótrofa, incluíndo aspectos como a estequiometría, parámetros cinéticos e moleculares, así como a bioquímica do proceso. Tamén se inclúen os efectos de diferentes factores (temperatura, pH, inhibidores, relación S/N, etc.) e a descrición dos diferentes microorganismos capaces de levar a cabo o proceso de desnitrificación autótrofa. Finalmente preséntase a aplicabilidade do proceso.

Capítulo 2. Neste capítulo descríbense os métodos analíticos empregados nesta tese. Inclúense os métodos fisicoquímicos comunmente empregados para analizar as augas residuais (análise de compostos nitroxenados, pH, sólidos, etc.) e os métodos empregados para caracterizar a biomasa (índice volumétrico de lamas e actividades específicas). A evolución e identificación das diferentes poboacións microbianas presentes na biomasa foron estudadas por medio de técnicas moleculares como: FISH, DGGE, PCR e secuenciación.

Capítulo 3. Neste capítulo levouse a cabo o enriquecemento da biomasa desnitrificante autótrofa, para o seu posterior emprego como inóculo dos reactores. A actividade inicial foi de 9,5 mg $S_2O_3^{2-}$-S (g SSV)$^{-1}$ d^{-1}. Coa finalidade de manter unha elevada retención da biomasa, elixiuse un sistema de reacción descontinuo para levar a cabo o enriquecemento da biomasa desnitrificante autótrofa.

Despois dunha serie de alimentacións sucesivas cun medio sintético con tiosulfato e nitrato, acadouse o enriquecemento da biomasa desnitrificante autótrofa. Despois de 95 días a súa actividade específica foi 187 mg $S_2O_3^{2-}$-S (g SSV)$^{-1}$ d^{-1}.

Ao final do experimento, a biomasa obtida foi capaz de empregar diferentes compostos de xofre como doadores de electróns para levar a cabo o proceso de

desnitrificación autótrofa. As actividades específicas obtidas foron 211, 153 e 11,5 mg NO_3^--N (g SSV)$^{-1}$ d^{-1} empregando tiosulfato, sulfuro e xofre elemental, respectivamente.

Capítulo 4. Neste capítulo levouse a cabo a desnitrificación autótrofa nun reactor SBR. O reactor foi operado durante 220 días, cunha velocidade de carga de sulfuro (SLR) de 200 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$ ata 450 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$. As eficacias de eliminación de sulfuro e nitrato foron de 100% e 67%, respectivamente. A eficacia do reactor viuse seriamente afectada polo valor do pH. Valores superiores a 9,0 causaron que o sulfuro fora oxidado só a xofre elemental, que se acumulou no interior do reactor. Eses valores de pH tamén causaron un descenso do 10% na actividade específica da biomasa. Só cando o pH se mantivo controlado nun valor arredor de 8 foi posible a recuperación da capacidade do sistema para oxidar completamente o sulfuro a sulfato.

Capítulo 5. Neste capítulo empregouse un sistema combinado de nitrificación/desnitrificación autótrofa para o tratamento dun efluente procedente dun dixestor anaerobio, o cal trataba as augas residuais xeradas nunha industria procesadora de peixe. O reactor nitrificante foi capaz de oxidar a nitrato una velocidade de carga nitroxenada (ALR) de 314 mg NH_4^+-N$\cdot L^{-1}\cdot d^{-1}$ cunha eficacia do 100%. O efluente dese reactor foi alimentado a un reactor desnitrificante autótrofo que trataba unha máxima carga de sulfuro aplicada de 200 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$, cunha eficacia do 100%. Sen embargo só se eliminou o 30% do nitrato alimentado debido á limitación por sulfuro. Polo tanto a adición de xofre elemental proponse como unha alternativa para mellorar a eliminación de nitrato neste sistema.

O custo de operación estimado para este sistema é de 0,80 €/Kg $N_{eliminado}$, que é inferior ó calculado para o sistema convencional de nitrificación/desnitrificación. Sen embargo o sistema SHARON/anammox é o proceso máis económico para tratar efluentes con baixas relacións C/N. Unha vantaxe do sistema combinado de nitrificación e desnitrificación autótrofa (empregando xofre elemental) podería ser a súa maior estabilidade fronte ó proceso SHARON/anammox.

Capítulo 6. Neste capítulo estudáronse os aspectos cinéticos do proceso de desnitrificación autótrofa.

Os resultados obtidos a diferentes concentracións de sulfuro/nitrato mostran que o nitrato ten un efecto inhibitorio sobre a desnitrificación a concentracións superiores a 200 mg NO_3^--N$\cdot L^{-1}$. Empregouse o modelo de Haldane para explicar este comportamento, obténdose os seguintes parámetros cinéticos: V_{max}= 0,470 ± 0,0013 mg $N_2\cdot L^{-1}\cdot min^{-1}$, Ks= 13,51 ± 0,113 mg NO_3^--N$\cdot L^{-1}$ e K*i*= 355 ± 4,38 mg L^{-1}. Tamén se observou inhibición por

nitrito y esta ocorreu a concentracións catro veces inferiores ás atopadas para o nitrato. O valor de IC_{50} (concentración que produce o 50% de inhibición) obtido foi de 48 mg NO_2^--N L^{-1}.

Os valores óptimos atopados de temperatura e pH para levar a cabo a desnitrificación autótrofa foron 35 ºC e 8,0 respectivamente.

Non se atoparon efectos inhibitorios causados por amonio, amonio libre e fosfato ás distintas concentracións empregadas. Son necesarios máis estudos acerca dos posíbeis efectos destes compostos.

Capítulo 7. Neste capítulo analizáronse as comunidades microbianas da biomasa desnitrificante autótrofa por medio de técnicas moleculares (FISH, PCR, DGGE e secuenciación). Os resultados obtidos mediante a aplicación da técnica FISH revelaron que o inóculo carecía de bacterias desnitrificantes autotróficas, o cal explica a baixa actividade desnitrificante ao comezo deste traballo. Sen embargo a biomasa enriquecida e a biomasa presente nos reactores operados nesta tese estiveron caracterizadas por unha elevada densidade de bacterias identificadas como *Thiobacillus denitrificans*. Esta poboación é moi sensíbel aos cambios de pH, o cal explica a baixa eficacia do sistema cando o pH acadou valores arredor de 9. Non se observou un cambio na poboación cando o medio de alimentación sintético foi trocado polo efluente nitrificado dun reactor anaerobio que trataba augas procedentes da industria conserveira.

A análise filoxenética mostrou que a comunidade microbiana que formaba as lamas está caracterizada por bacterias do tipo *Thiobacillus denitrificans* e *Bacteroidetes* principalmente, ademais doutros dous grupos de bacterias capaces de empregar algunha fonte de xofre, *Chlorobi* e un candidato da división JS1, así como un grupo de bacterias semi-anaerobias capaces de empregar nitrato e relacionadas con *Stenotrophomonas*.

Chapter 1.

Introduction

Summary

In this chapter a brief compilation of the state of the art of the research and applications on autotrophic denitrification is presented. Initially a description of the way the anthropogenic activities have altered the natural sulphur cycle, affected aquatic and terrestrial environments and caused atmospheric and health problems is provided. Then after introducing the problem of sulphur pollution, different biological processes for sulphur compounds removal are analysed paying special attention to the autotrophic denitrification. In this process nitrate and/or nitrite are converted to nitrogen gas by means of reduced sulphur compounds which act as electron donors. At the same time inorganic carbon is used for grow and less sludge production occurs. At the end of the chapter a brief description of the main reactor configurations used to carry out the autotrophic denitrification is presented and the main operational conditions, the used sulphur compounds and the obtained removal efficiencies are summarized.

Part of this chapter has been included in:

Fajardo *et al*. (2008). Depuración conjunta de aguas ricas en nitratos y efluentes con compuestos reducidos de azufre. RETEMA, 127:38-50.

Campos *et al*. (2010). Chapter Novel Biological Nitrogen-Removal Processes: Applications and Perspectives. In Fluid Waste Disposal. Environmental Science, Engineering and Technology Series. New York. 153-181.

1.1. INTRODUCTION

Anthropogenic activities have contributed to the imbalance of nitrogen and sulphur natural cycles. Furthermore many negative effects in nature are associated to the emissions of the sulphur compounds and their transformations. Hydrogen sulphide, known for its toxicity, corrosive properties and bad odour even at very low concentrations, causes serious health problems such as coma and unconsciousness at concentrations of 200 and 300 $mg \cdot L^{-1}$ (Borkenstein and Fischer*.,* 2006; Syed *et al.,* 2004). The combustion of sulphide produces sulphur dioxide (SO_2), which contributes to the acid rain and soil acidification (Borkenstein and Fischer, 2006; Syed *et al.,* 2006; van den Bosch *et al*., 2007; Mahmood *et al*.. 2007).

Effluents produced in certain industrial sectors are rich in compounds containing sulphur in reduced or oxidised forms (textile, tanning, food processing, pulp and paper, petrochemical industries, etc.) (Table 1.1). Also during the anaerobic treatment of such wastewaters high concentrations of reduced sulphur compounds such as sulphide are generated (Tandukar *et al*., 2009; Janssen *et al*., 1999; Krishnakumar *et al*., 2005). The sulphide causes several problems in conventional activated sludge plants. Concentrations higher than 0.5 $mg \cdot L^{-1}$ have toxic effects on the nitrifying activity, favour the growth of filamentous bacteria and the floc disruption. On the other hand, the required high oxygen demand for its oxidation (2 mol O_2 (mol $S^{2-})^{-1}$) provokes an increase of the operational costs (Vaninni *et al*., 2006; Mahmood *et al.,* 2007).

In the industrial sector there is another group of industries which generate effluents characterized by high nitrogen concentrations and low organic matter content. In these cases external addition of the organic carbon source is required to carry out nitrogen removal causing the increase of the operational costs.

In some cases the combination of the effluents containing nitrogen and sulphur compounds is of interest to perform the autotrophic denitrification process which combines the nitrogen and sulphur compounds to produce nitrogen gas while the sulphur compounds are oxidized. This process can be used to denitrify nitrate and/or nitrite from those effluents with low or none organic matter content by simply combining those ones with effluents containing reduced sulphur compounds.

Table 1.1 Industrial wastewaters containing high concentration of sulphur compounds (Adapted from Tandukar *et al*., 2009)

Wastewater	Sulphur compound (mg L^{-1})	Reference
Latex	100 -4000 (H_2S)	Rattanapan *et al.* (2009)
Crude oil processing	~150 (S^{2-})	Sekoulov and Brinke-Seiferth (1999)
Petroleum refinery	~150 (S^{2-})	Altas and Buyukgungor (2008)
Rubber latex	1819 (SO_4^{2-})	Kanyarat and Chaiprapat (2008)
Landfill leachate	1100 – 3800 (SO_4^{2-})	Tait *et al*. (2009)
Chemical industry	12000 – 35000 (SO_4^{2-})	Silva *et al*. (2002)
Molasses	1800 (SO_4^{2-})	Percheron (1997)
Tannery	750 – 1250 (SO_4^{2-}); 140 -280 (S^{2-})	Genswchow *et al.* (1996)
Pulp and paper	50 – 200 (S^{2-}); 1200 – 1500 (SO_4^{2-})	Lens *et al*. (1998)
Beamhouse	500 – 1500 (S^{2-})	Lens *et al*. (1998)
Photographic	45500 ($S_2O_3^{2-}$); 8000 (SO_4^{2-}); 3500 (SO_3^{2-})	Lens *et al*. (1998)
Fish cannery	300 - 1000 (SO_4^{2-})	Kleerebezem and Méndez (2000)

Nowadays the society demands for the implementation of sustainable wastewater treatment systems that provide effluent quality, at low operational costs, with minimum area requirements and the potential of nutrients recovery. Apart from organic matter industrial effluents may also contain important amounts of nutrients such as nitrogen or sulphur which must be removed because they have important potential impacts on the environment. The biological processes involved in the natural sulphur cycle can be used in the wastewater treatment plants to remove sulphur compounds from the liquid effluents. In the following sections a description of the most important ones is performed.

1.2. SULPHUR IN NATURE

In nature sulphur compounds are found as calcium sulphate (gypsum), in the form of metal complexes (pyrite) and elemental sulphur (Lens and Kuenen, 2001).

The sulphur cycle involves both oxidation and reduction processes. In the reduction processes the oxidized sulphur compounds (sulphate, sulphite and elemental sulphur) are biologically reduced, while in the oxidation processes both chemical and biological processes are involved (Figure 1.1).

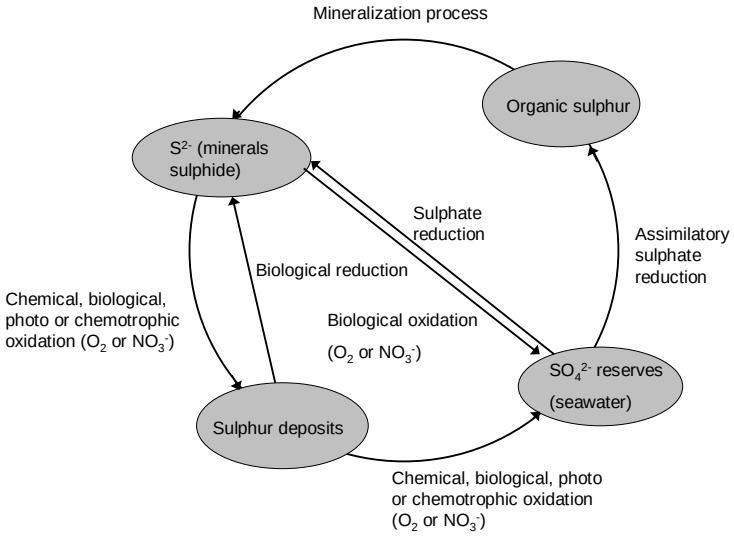

Figure 1.1 Sulphur cycle (adapted from Lens and Kuenen, 2001).

During the reduction processes sulphate reducing bacteria (SRB) use oxidized sulphur compounds (SO_4^{2-}, $S_2O_3^{2-}$, SO_3^{-2}) as terminal electron acceptor and organic compounds, such as lactate, ethanol or propionate, or H_2 as electron donor. This process can be applied to remove high concentrations of sulphate or sulphite. Microbiological and enzymatic aspects of SRB have been widely studied (Takakuma S., 1992; Muyzer G. and Stams A., 2008). The main representative micro-organisms include members of genus *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfovibrio Desulfomonas* etc. (Jansen *et al*., 2001; Lens and Kuenen, 2001).

The biological sulphur oxidation is carried out by phototrophic oxidation or chemolithotrophic oxidation. During the phototrophic oxidation green and purple sulphur bacteria oxidize sulphide to elemental sulphur and sulphate under limiting sulphide conditions. This process requires only light, CO_2 and inorganic nutrients for growth and it occurs under anaerobic conditions. It has been performed in different types of reactors with high sulphide removal efficiencies (Syed *et al*., 2006).

During chemolithotrophic oxidation the reduced sulphur compounds can be partially oxidizing to elemental sulphur under oxygen limiting conditions (equation 1.1) or fully oxidized to sulphate using excess of oxygen by aerobic bacteria of the genus *Thiobacillus* (equation 1.2) (Jansen *et al*., 2001).

$$H_2S + 0.5\ O_2 \rightarrow S^o\downarrow + H_2O \qquad [1.1]$$

$$H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2H^+ \qquad [1.2]$$

In nature there are a number of micro-organisms that can combine the nitrogen and the sulphur cycles. These micro-organisms play a significant role in marine ecosystems, mainly in the limit between anoxic and aerobic interfaces (Robertson and Kuenen, 1992; Fdz-Polanco *et al*., 2001). The combination of both cycles implicates the oxidation of the reduced forms of sulphur and the reduction of oxidized nitrogen compounds such as nitrate or nitrite in the autotrophic denitrification process (Figure 1.2).

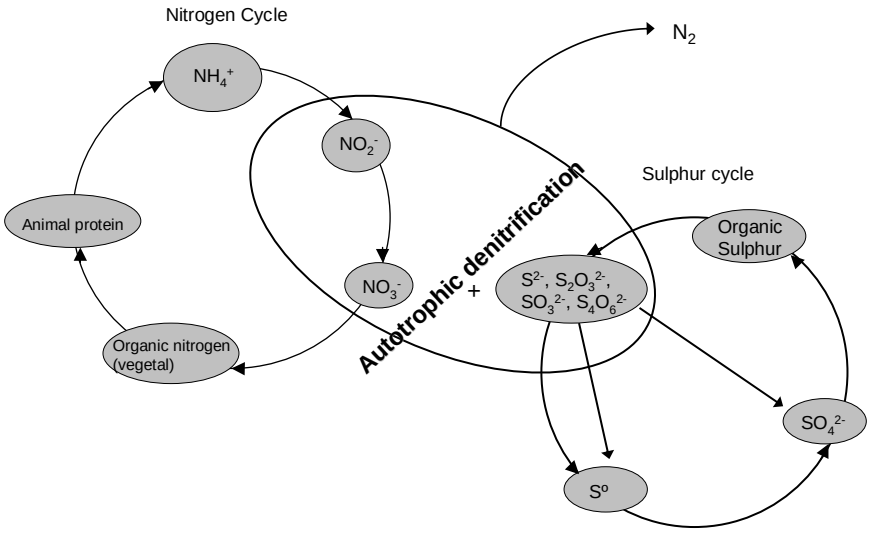

Figure 1.2 Coupling between nitrogen and sulphur cycles.

1.3. AUTOTROPHIC DENITRIFICATION

The autotrophic denitrification process is the reduction of NO_3^- and/or NO_2^- to N_2, with H_2 or sulphur compounds ($S_2O_3^{2-}$; S^{2-}; S^o, $S_4O_6^{2-}$; SO_3^{2-}) as electrons donors, CO_2 and HCO_3^- as carbon source and by the action of sulphur oxidizing bacteria (SOB) (Koenig *et al.,* 2005; Fernández *et al.,* 2006).

1.3.1. Sulphur Oxidizing Bacteria (SOB)

The sulphur oxidizing bacteria are a heterogeneous group, whose members have the ability to use reducing sulphur compounds as energy source. These bacteria are chemolithotrophic obligate or facultative, and can grow in mesophillic and thermophilic environments (Robertson and Kuenen, 1992; Krishnakumar and Manilal, 1999).

In nature, these bacteria are often found in the anoxic and aerobic of sediments and water interface, where the concentrations of sulphur compounds are also low, in

geothermal vents, in UASB reactors and in activated sludge systems (Robertson and Kuenen, 1992; Krishnakumar and Manilal, 1999; Gadekar *et al.,* 2006).

There are many micro-organisms able to use sulphur compounds such as sulphate reducing bacteria (*Desulfovibrio, Desulfobacter, Desulfococcus*…) but few microorganisms are able to carry out the autotrophic denitrification (autotrophic denitrifying bacteria) using reduced sulphur compounds as electron donor and nitrate or nitrite as electron acceptor.

Autotrophic denitrifying bacteria are member of phylum *Proteobacteria* (Figure 1.3). The micro-organism best studied, able to carry out autotrophic denitrification using reduced sulphur compounds ($S_2O_3^{2-}$, S^{2-} and Sº), is *Thiobacillus denitrificans* (*β-Proteobacteria* class) and it is known as colourless sulphur bacteria (Robertoson and Kuenen, 1992). It is a rod-shaped, gram-negative with polar flagella motile or non-motile bacteria and it grows under mesophilic conditions. *Thiobacillus thiophilus* has been recently also reported as an autotrophic denitrifying bacterium which uses thiosulphate and nitrate (Kellermann and Griebler, 2009).

Another major bacterium performing the autotrophic denitrification is *Sulfurimonas denitrificans* (*Epsilonproteobacteria*). It is a rod-shaped no motile bacteria and it is able to oxidize $S_2O_3^{2-}$ and HS^- coupled to the reduction of nitrate into nitrogen gas (Takai *et al.,* 2006; Tandukar *et al*., 2009; Gadekar *et al*., 2006).

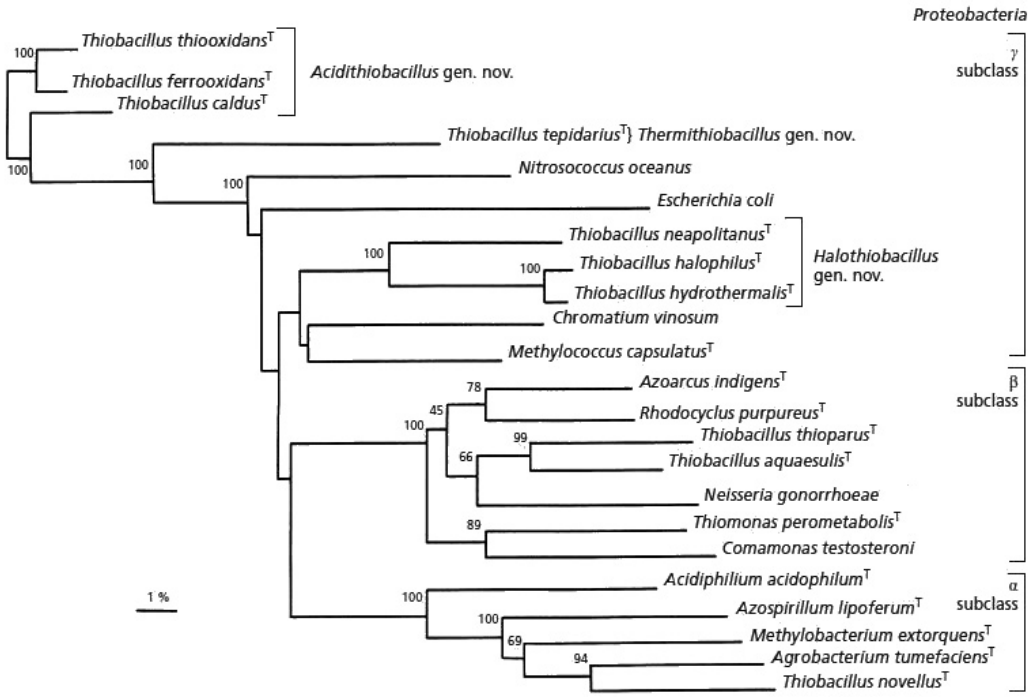

Figure 1.3 Phylogenetic tree based in 16S rRNA for members of *Thiobacillus* genera (Kelly and Wood, 2000).

1.3.2. Kinetics of Sulphur Oxidizing Bacteria

The majority of the kinetic studies have been conducted with pure cultures of *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*. The estimated kinetic parameters from the different studied bacterial populations present a wide range of values (Table 1.2) which indicates their large diversity.

Table 1.2 Kinetic parameters of sulphur oxidizing bacteria.

	μ_{max} (h^{-1})	r_{max} (h^{-1})	K_s (mg $N \cdot L^{-1}$)	Y (mg VSS·(mg NO_3^--N) $^{-1}$)	Reference
Enriched sludge	0.12-0.2	0.3-0.4	3-10	0.4-0.5	Oh *et al*. (2000)
Thiobacillus denitrificans	0.11		0.2	0.4-0.57	Claus *et al.* (1985)
Thiomicrospira denitrificans	0.19-0.22	0.36	0.22*	0.5**	Gadekar *et al.* (2006)
Thiobacillus denitrificans	0.02-0.08				Justin and Kelly (1978)
Enriched sludge	0.006		0.398	0.81-1.1	Zeng and Zang (2005)

*mg $S \cdot L^{-1}$ **mg VSS·(mg S^{-2}-S) $^{-1}$

1.3.3. Biochemistry of autotrophic denitrification

During the autotrophic denitrification two simultaneous reactions are performed:, the nitrate reduction to nitrogen gas and the oxidation of reduced sulphur compounds to sulphate, these processes involve a series of enzymes for the denitrifying and sulphur oxidizing pathways, closely interrelated (Figure 1.4).

In the denitrifying pathway four steps catalyzed by enzymes are recognized (Moura and Moura, 2001):

a) Reduction of nitrate to nitrite catalysed by nitrate reductase (*Nar*)

b) Nitrite reduction to nitric oxide catalyzed by nitrite reductase (*Nir*)

c) Reduction of nitric oxide to nitrous oxide catalyzed by nitric oxide reductase (*Nos*)

d) Reduction of nitrous oxide to nitrogen gas catalyzed by nitrous oxide reductase (*N2or*)

In the sulphur oxidation pathway the oxidation of reduced sulphur compounds (S^{2-}, $S_2O_3^{2-}$ or S^o) to SO_4^{2-} is performed. In the case of the thiosulphate oxidation, three pathways are recognized (Friedrich *et al.,* 2001; Brüser *et al*., 2000; Meyer *et al*., 2007):

a) Oxidation of thiosulphate to sulphate by means of a thiosulphate dehydrogenase and tetrathionate hydrolase.

b) Oxidation of thiosulphate to sulphate by means of a multienzyme complex (*Sox*-MC).

c) Oxidation of thiosulphate to sulphate with elemental sulphur as intermediate product by a thiosulphate cleaving enzyme, by means of s-oxygenase (*S-ox*) and sulphite oxidase (Sul-ox) enzymes.

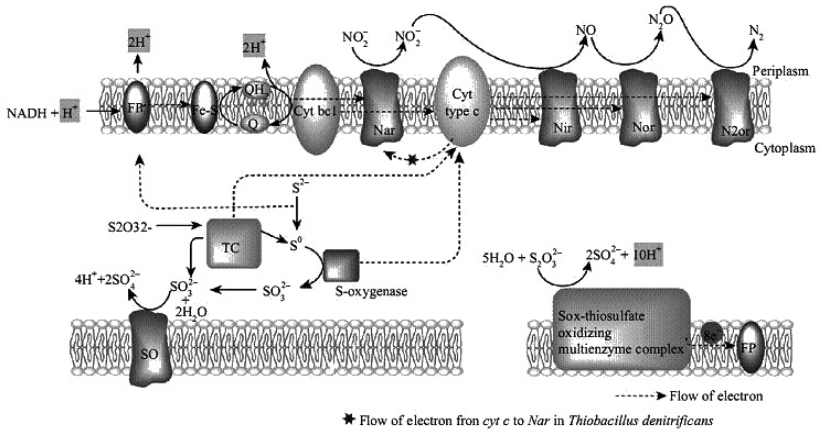

Figure 1.4 Enzymatic systems involved in the simultaneous denitrification and sulphur oxidation during the autotrophic denitrification process (Tandukar *et al*., 2009).

1.3.4. Key operational parameters for autotrophic denitrification

There are some basic operational parameters to consider during the application of autotrophic denitrification for the treatment of wastewaters containing sulphur and nitrogen compounds such as:

pH

As it is the case with all micro-organisms, the autotrophic bacteria have an optimum pH range of operation. In the case of *Thiobacillus denitrificans* it is 7-8 (Oh *et al*., 2000; Claus and Kutzner, 1985). In this range of pH values the end products of denitrification are N_2 and sulphate while at pH values below 7 the denitrification is incomplete and intermediate products such as nitrite and/or elemental sulphur are detected. At pH values under 6 or over 9 the complete inhibition of denitrification is observed (Moon *et al*., 2004; Oh *et al*., 2000).

Temperature

Sulphur oxidizing bacteria have been found in mesophilic environments (25 - 35 º C), their optimum value of temperature being around 35 ºC. When temperature is higher than 40 ºC (Oh *et al*., 2000) or lower than 15 ºC (Yamamoto-Ikemoto *et al*., 2000), the autotrophic denitrification rate is negligible.

Alkalinity

A source of alkalinity as HCO_3^- is necessary to neutralize the protons produced during the biological reaction and to provide a carbon source for bacterial growth. According to equations 1.3, 1.4 and 1.5, alkalinity (as $CaCO_3$) consumption per gram of NO_3^--N removed (alkalinity consumption ratio) is 2.49, 0.93 and 4.57 when $S_2O_3^{2-}$, H_2S and Sº, respectively, are used.

$$0.844\ S_2O_3^{2-} + NO_3^- + 0.347\ CO_2 + 0.086\ HCO_3^- + 0.086\ NH_4^+ + 0.434\ H_2O \quad [1.3]$$
$$\rightarrow 1.689\ SO_4^{2-} + 0.5\ N_2 + 0.086\ C_5H_7O_2N + 0.697\ H^+$$

$$0.421\ H_2S + 0.421\ HS^- + NO_3^- + 0.346\ CO_2 + 0.086\ HCO_3^- + 0.086\ NH_4^+ \quad [1.4]$$
$$\rightarrow 0.842\ SO_4^{2-} + 0.5\ N_2 + 0.086\ C_5H_7O_2N + 0.434\ H_2O + 0.262\ H^+$$

$$1.1\ S^o + NO_3^- + 0.76\ H_2O + 0.4\ CO_2 + 0.08\ NH_4^+ \quad [1.5]$$
$$\rightarrow 1.1\ SO_4^{2-} + 0.5\ N_2 + 0.08\ C_5H_7O_2N + 1.28\ H^+$$

Many researches have been performed using calcite ($CaCO_3$), as alkalinity source for autotrophic denitrification, in packed reactors containing sulphur as electrons donor. In these cases the alkalinity value ranged between 85 – 95 mg $CaCO_3 \cdot L^{-1}$ at pH values of 6.9-7.5 (Moon *et al*., 2004; Kim and Bae, 2000).

S/N ratio

It has been observed that the S/N ratio of the feeding plays an important role on the autotrophic denitrification. Oh *et al*. (2000) tested different S/N ratios with thiosulphate as electron donor and nitrate as electron acceptor and they found that at ratios below of 6.51 g S/g N (sulphur limitation) the denitrification was only carried out to nitrite. In the case of S/N ratios higher than the stoichiometric one, the sulphur compound is only oxidized to elemental sulphur (Gadekar *et al*., 2006). The control of the inlet S/N ratio is required to drive the process to the products of interest.

Sulphur compounds

The denitrification rate depends on the oxidation state of the sulphur compound present in the wastewater (Table 1.3). The oxidation rates of thiosulphate are around 212 mg NO_3^--N·g VSS^{-1}·d^{-1} which are ten times higher than that of 22 mg NO_3^--N·g VSS^{-1}·d^{-1} reported elsewhere using elemental sulphur (Beristain *et al*., 2006).

Table 1.3 Oxidation-reduction reactions with different sulphur compounds.

Reaction	$\Delta G^{o'}$ (kJ·mol^{-1})	Reference
$1.25\ S^{2-} + 2\ NO_3^- + 2\ H^+ \rightarrow 1.25\ SO_4^{2-} + N_2 + H_2O$	-972.8	Reyes-Avila *et al.* (2004)
$5\ S^{2-} + 2\ NO_3^- + 12\ H^+ \rightarrow 5\ S^o + N_2 + 6\ H_2O$	-1151.38	Wang *et al.* (2005)
$5\ S^o + 6\ NO_3^- + 8\ H_2O \rightarrow 5\ H_2SO_4 + 3\ N_2 + 6\ OH^-$	-1833.96	
$S^o + 1.2\ NO_3^- + 0.4\ H_2O \rightarrow SO_4^{2-} + 0.6\ N_2 + 0.8\ H^+$	-547.6	Beristain *et al.* (2006)
$S_2O_3^{2-} + 1.6\ NO_3^- + 0.2\ H_2O \rightarrow 2\ SO_4^{2-} + 0.8\ N_2 + 0.4\ H^+$	-765.7	

Oxygen

Oxygen and nitrate are electron acceptors for the oxidation of sulphur compounds. The oxidation of sulphur compounds in the presence of oxygen is thermodynamically more favoured than the oxidation using nitrate. Therefore, its presence should be avoided. Several research works agree that the minimum concentration of dissolved oxygen which does not cause inhibition on autotrophic denitrification is between of 0.1-0.3 mg O_2·L^{-1}.

Above these concentrations denitrification is inhibited (Sublette *et al*., 1998; Kimuara *et al*., 2002; Gu *et al*., 2004).

Presence of inhibitory compounds

It has been reported the inhibition of the denitrification by nitrate at concentrations of 660 mg NO_3^--N·L^{-1}, while nitrite appears to be a strong inhibitor of the denitrification even at low concentrations (36-60 mg NO_3^--N·L^{-1}) (Oh *et al*., 2002). The inhibition of denitrification by heavy metals such as Zn and Cu at concentrations of 0.5 and 1.0 mg·L^{-1} has also been reported (Claus and Kutzner, 1985; Krishnakumar and Manilal, 1999; Oh *et al*., 2000; Moon *et al*., 2006). The organic matter has no inhibitory effect on the process but it affects the oxidation of sulphur species, decreasing the formation of sulphate (Kim and Son, 2000; Oh *et al.,* 2002). The sulphate is a product of the process and it has been reported to provoke partial inhibition at concentrations of 500 mg SO_4^{-2}-S·L^{-1} and total activity depletion at 6400 mg SO_4^{-2}-S·L^{-1} (Campos *et al*., 2008; Claus and Kutzer, 1985).

1.4. ADVANTAGES AND DISADVANTAGES

Even taking into account that the autotrophic denitrification is a good option for simultaneous removal of nitrogen and sulphur compounds it has certain advantages and disadvantages. Between the advantages the following ones are found:

a) Many wastewaters have a low organic matter concentration; therefore, the addition of an external carbon source is necessary to remove nitrogen by heterotrophic denitrification. However, if autotrophic denitrification is applied, elemental sulphur can be used as electron donor. As this compound is cheaper (0.25$·(kg NO_3^--N)$^{-1}$) than methanol (1.3-3.6$·(kg NO_3^--N)$^{-1}$) and acetic acid (2.5$·(kg NO_3^--N)$^{-1}$) a reduction of the operational costs can be obtained.

b) Due to the fact that only a few micro-organisms are able to use HS^-, $S_2O_3^{2-}$ or Sº as the electron donor for grow, a strong populations selection is carried out and, furthermore, low sludge production is obtained.

Among the disadvantages the following aspects must be taken into account:

a) Although many wastewaters contain high concentrations of sulphur and nitrogen compounds in different forms, the former is in many cases as sulphide which provokes the

inhibition of the nitrification at low concentrations, 0.5 mg $HS^{-}{\cdot}L^{-1}$ (Vaninni *et al*., 2006), while the latter is mainly in form of ammonia which should be first nitrified. Special attention must be then paid to avoid the presence of the sulphide in the nitrification reactor.

b) When wastewaters with high nitrate concentrations are treated, effluents with high sulphate concentrations are generated. In these cases, the development of anaerobic zones could lead to the generation of sulphide (Oh *et al.,* 2001).

c) Presence of sulphur compounds in the wastewaters can cause sludge bulking by proliferation of filamentous bacteria.

1.5. TECHNOLOGIES TO CARRY OUT THE AUTOTROPHIC DENITRIFICATION PROCESS

Autotrophic denitrification has been extensively studied in laboratory scale systems. Several kinds of reactors have been used such as reactors packed with sulphur or sulphur/limestone (Figure 1.5a), biofilm reactors, continuous stirred tank reactors (CSTR) (Figure 1.5b), Upflow anoxic sludge blanket (UASB), Upflow anoxic filter (UAF) and activated sludge systems. In many cases high removal efficiencies of both nitrate and sulphur species were obtained (Table 1. 4).

Table 1.4 Autotrophic Denitrifying Reactors.

Reactor	Bacterial population	Electron donor	Bedron acceptor	Product	Removal efficiency	Reference
CSTR	*Thiomicrospira* sp.	HS^-	NO_3^-	SO_4^{2-}, NO_2^-	100% HS^-	Gadekar *et al.* (2006)
CSTR	*T. denitrificans*	S^{2-}	NO_3^-	S°	100% S^{2-}, 95% NO_3^-	Wang *et al.* (2005)
Fed Batch	Enriched sludge	H_2S, $S_2O_3^{2-}$, S°	NO_3^-	SO_4^{2-}, N_2, NO_2^-	100% N and S	Beristain *et al.* (2006)
Packed bed reactor (S°)	Enriched sludge	S°	NO_3^-	SO_4^{2-}	95% NO_3^-	Soares (2002)
Membrane reactor	Enriched sludge	S°	NO_3^-	SO_4^{2-}	80% NO_3^-	Kimura *et al.* (2002)
Fluidized bed reactor (S°)	Enriched sludge	S°	NO_3^-	SO_4^{2-}	90% NO_3^-	Kim *et al.* (2004)
UAF	Enriched sludge	HS^-	NO_3^-	SO_4^{2-}	100% HS^-, NO_3^-	Kleerebezem and Méndez (2002)
Packed bed reactor (S°)	*T. denitrificans*	S°	NO_3^-	SO_4^{2-}, H_2S, NO_2^-	97% NO_3^-	Gu *et al.* (2004)
Packed bed reactor (S°)	*T. denitrificans*	S°	NO_3^-	SO_4^{2-}	100% NO_3^-	Moon *et al.* (2004)
CSTR	Bioaugmented *T. denitrificans*	HS^-	NO_3^-	SO_4^{2-}	100% HS^-	Manconi *et al.* (2007)
CSTR	Bioaugmented *T. denitrificans*	$S_2O_3^{2-}$	NO_3^-	SO_4^{2-}	95% $S_2O_3^{2-}$, 100% NO_3^-	Manconi *et al.* (2007)
UASB	Methanogenic sludge	HS^-	NO_2^-	SO_4^{2-}, S°, N_2	98% HS^-, 82% NO_2^-	Mahmood *et al.* (2008)
UAF		$S_2O_3^{2-}$	NO_3^-	SO_4^{2-}, S°, N_2	90% NO_3^-	Yamamoto-Ikemoto *et al.* (2000)

The first reported research with autotrophic denitrification has been performed in a packed bed reactor with S° and S°/limestone known as Sulphur:limestone autotrophic denitrification reactor (SLAD) (Kuai and Verstraete, 1999; Zhang and Lampe, 1999; Koenig and Liu, 2001). In this system both sulphur and limestone are used as carrier for biomass and limestone also provides alkalinity to maintain a suitable value of pH (Zhang and Lampe, 1999; Kim *et al.,* 2004). Nitrogen loading rates (NLR) up to 2000 mg NO_3^--N·L^{-1}·d^{-1} can be treated by this kind of systems (Koenig and Liu 1996; Flere, 1999; Kim *et al*., 2000, 2004).

However the packed bed reactors can present clogging problems due to the excess of biomass which provokes the gas entrapment, limiting the mass transfer of nitrate from bulk liquid to biofilm (Flere, 1999; Kim *et al*., 2004). On the other hand, the size of the elemental sulphur particles affect both nitrogen loading rate removed and nitrate removal efficiency and it is recommended to use particles sizes between 2 and 5 mm to optimize the performance of the system (Sierra-Alvarez *et al*., 2007; Koenig and Liu, 1996, 2001; Moon *et al*., 2004, 2006, 2008).

Most of the studies carried out using thiosulphate has been done in batch reactors in order to obtain the kinetic parameters of the biomass (Claus and Kutzner, 1985; Justin and Kelly, 1978; Oh *et al*., 2000; Campos *et al*., 2008) and there are only few works where continuous autotrophic denitrifying reactors were operated using this sulphur source. Yamamoto-Ikemoto *et al*. (2000) and Fernández *et al.* (2008) were able to treat up to 6.3 g NO_3^-- N L^{-1}·d^{-1} with nitrogen removal efficiencies of 90% using an UASB system while Manconi *et al.* (2007) achieved to treat a NLR of 0.490 g NO_3^-- N·L^{-1}·d^{-1} an activated sludge system.

Autotrophic denitrification, using sulphide as sulphur source, was widely studied with different kinds of reactors: activated sludge reactors (Manconi *et al*., 2006, 2007); UASB (Mahmood *et al*., 2007, 2008; Jing *et al*., 2009; Fernández *et al.,* 2008); CSTRs (Gadekar *et al*., 2006; Reyes-Avila *et al*., 2004; Wang *et al*., 2005; Vaiopoulou *et al*., 2005); biofilm reactors (Kleerebezem and Méndez, 2002; Garcia de Lomas *et al*., 2007; Ma *et al*., 2006); and sequential batch reactors (Pérez *et al*., 2007). In these works the NLR applied ranged between 0.1 and 0.6 g NO_3^-- N·L^{-1}·d^{-1} while the SLR ranged between 0.3 – 3.25 g S^{2-}·L^{-1}·d^{-1}. Despite of high sulphide removal efficiencies can be obtained in these systems, their main disadvantage is the nitrite accumulation when high sulphide

concentrations are tested which provokes poor nitrogen removal efficiencies (Mahmood *et al.,* 2007; Manconi *et al*., 2007).

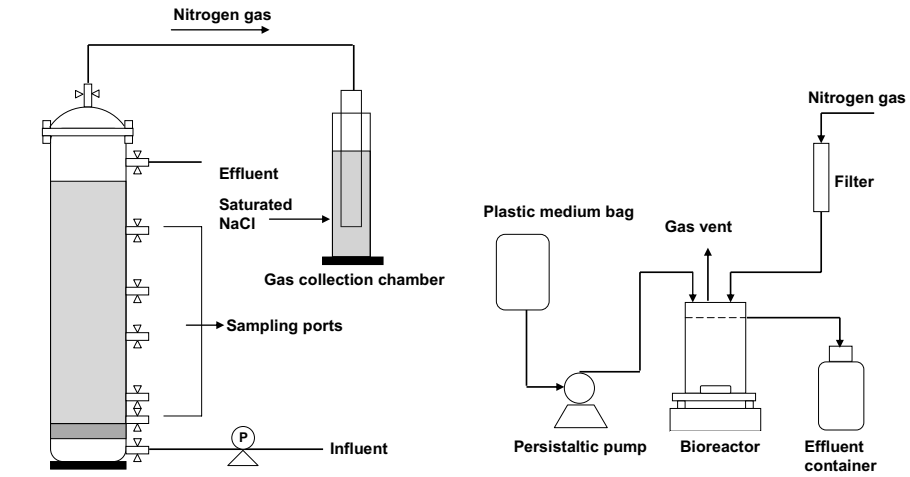

Figure 1.5 Configurations of denitrifying autotrophic reactors: a) packed reactor (S^0/limestone) (Moon *et al*., 2004) and b) CSTR (Gadekar *et al.,* 2006).

1.6. APPLICATIONS OF THE AUTOTROPHIC DENITRIFICATION PROCESS

1.6.1. Industrial wastewaters

The anaerobic treatment of effluents containing sulphate (canneries, petrochemical industries, tanneries, etc.) implies generation of sulphide. This compound presents some problems such as: a) odour problems and toxicity; b) decrease of organic matter removal efficiency and, therefore, less methane generated; c) corrosion problems; d) the need for biogas conditioning and postreatment of effluents. A simple method to remove sulphide is autotrophic denitrification and can be applied by using the ANANOX (Anaerobic-Anoxic-Oxic) technology (Garuti *et al.*, 2001).

This technology is based on a two units configuration. The first unit is an anaerobic reactor with three compartments (2 anaerobic + 1 anoxic) containing flocculent sludge (Figure 1.6). The second unit is a conventional activated sludge system with a settler. In the first unit, anaerobic digestion of organic matter and sulphate reduction into sulphide are carried out. During autotrophic denitrification, sulphide is again oxidized into sulphate with the nitrate coming from the effluent recirculated.

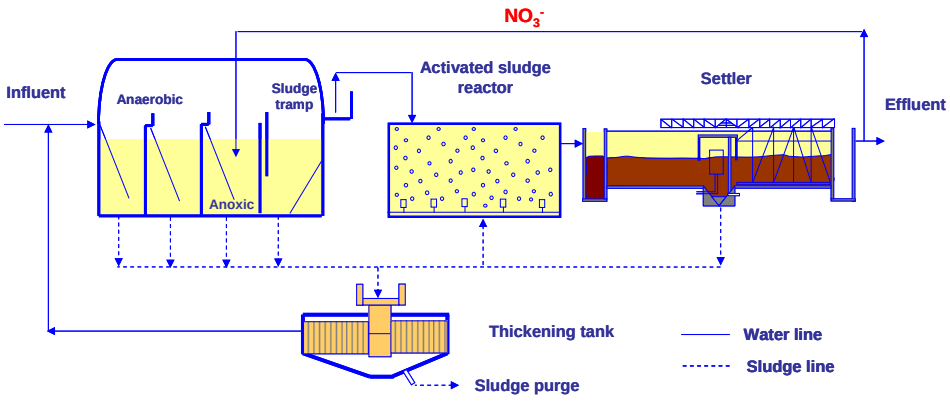

Figure 1.6 ANANOX process.

Kleerebezem and Méndez (2002) also proposed the use of sulphide to remove nitrate during the post-treatment of fish cannery effluents treated by anaerobic digestion. The proposed configuration presented several advantages compared to the conventional configuration (Figure 1.7) such as: 1) H_2S removal from biogas; 2) no requirement of recirculation between nitrifying and denitrifying reactors; 3) autotrophic denitrification produces less sludge and 4) it is no necessary to control the amount of organic matter in the effluent of the anaerobic digester to carry out heterotrophic denitrification.

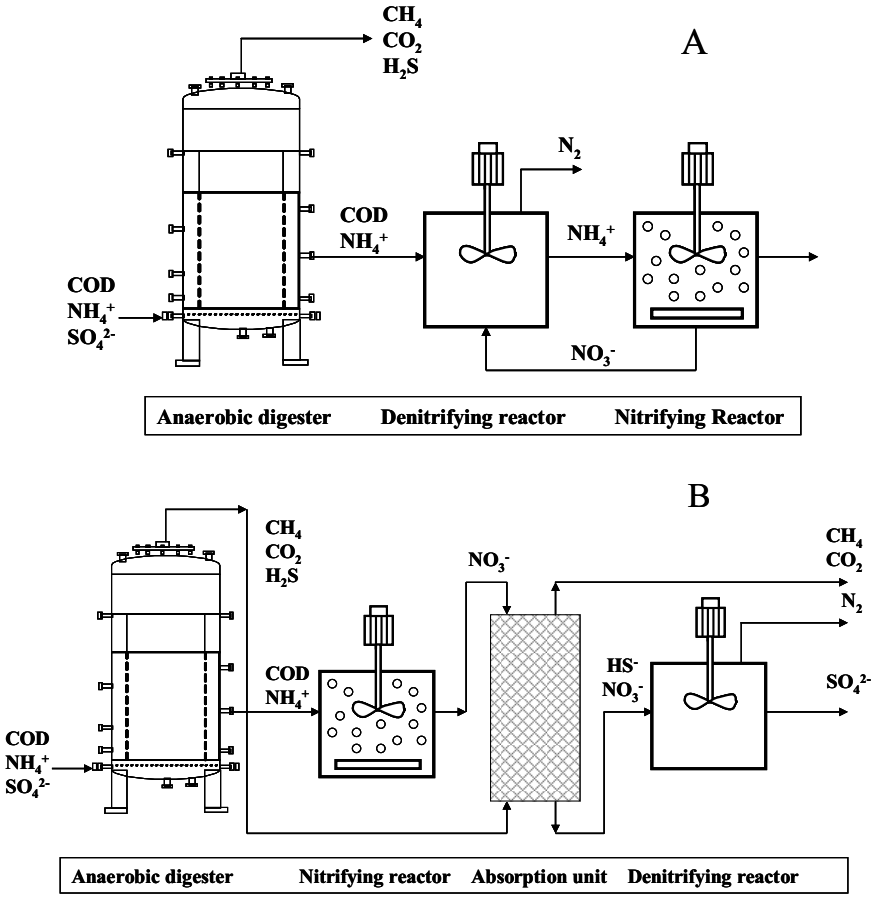

Figure 1.7 a) Conventional treatment of fish cannery effluents and b) Treatment of fish cannery effluents by applying autotrophic denitrification.

Vaiopoulou *et al*. (2005) applied the autotrophic denitrification process to remove both nitrate and sulphide from effluents of a petrochemical industry. According to the satisfactory obtained results during the operation of the pilot-scale plant, a new stage in the wastewater processing plant was introduced, reducing the CO_2 consumption by striping of H_2S from 2000 $m^3 \cdot h^{-1}$ to 600 $m^3 \cdot h^{-1}$ and obtaining considerable energy and financial savings.

1.6.2. Groundwater Bioremediation

In the last years, nitrate levels in ground waters exceeding the European Regulation (11.3 mg NO_3^--N·L^{-1}) were observed. The conventional method to remove nitrate is ionic exchange although the application at full scale of reverse osmosis also gave good results. Nevertheless, both processes generate a residual stream which needs a post-treatment. An alternative to these technologies is the denitrification. In the case of heterotrophic denitrification, organic matter (ethanol or methanol) must be added as electron donor that leads to a secondary contamination. This can be avoided if nitrate removal is done by autotrophic bacteria using elemental sulphur since it is not a toxic compound and it is insoluble in water. This process will generate sulphate and is recommended to apply to ground water with low endogenous sulphate levels to avoid sulphate concentrations higher than 400 mg SO_4^{-2}·L^{-1} (Flere and Zang, 1998; Moon *et al.,* 2004).

The application of autotrophic denitrification to ground water has been limited by the low biomass retention. Therefore, recent works are focused on combining this process with membrane (McAdam and Judd, 2006) or biofilm technologies (Soares, 2002) to achieve a complete retention of the biomass. The configurations proposed are the following (Figure 1.8):

(a) Bioreactor with extractive membrane: In this configuration, nitrate is extracted from water by molecular diffusion through the membrane to a stream containing both denitrifying biomass and electron donor (Figure1.8a).

(b) Bioreactor with filtration membrane: Denitrifying biomass is mixed with polluted ground water and electron donor. In this case, the membrane is used to separate biomass from treated water by application of pressure (Figure 1.8b).

(c) Biofilm reactor: Elemental sulphur particles could be used as both electron donor and support of autotrophic denitrifying biomass. A column filled with elemental sulphur granules and operated in an upflow mode could be a system very simple, stable and easy to maintain (Figure 1.8c).

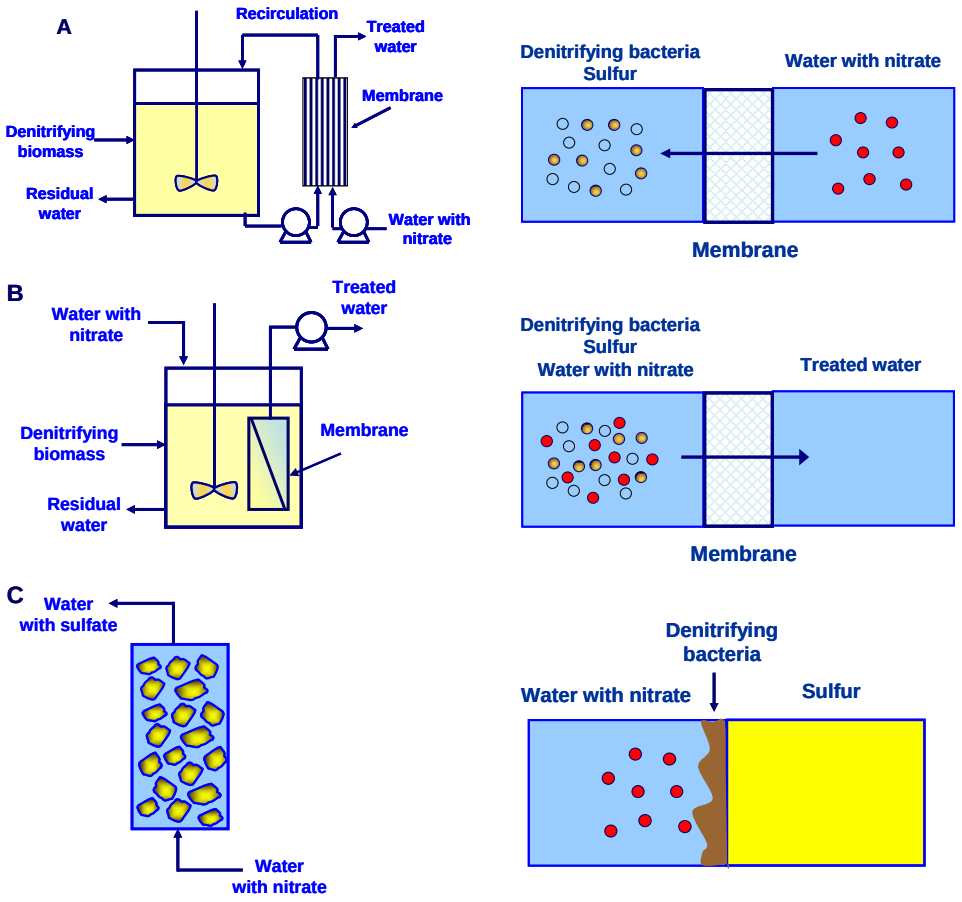

Figure 1.8 Systems to remove nitrate from ground water: a) bioreactor with extractive membrane, b) bioreactor with filtration membrane and c) biofilm reactor.

1.6.3. Nitrate Removal from Recirculating Aquaculture Systems

Factors such as limitations of water quality, land costs, disposal requirements and environmental impact are driving the aquaculture sector to more intensive practices. The use of recirculating systems allows reducing water used and disposed during aquaculture activities. Besides, it has another advantages: a) Saving of pumping costs; b) Control of pH and temperature which optimize fish production; c) Presence of pathogens is minimized which reduces mortality during the broodstock stage.

Since ammonia is toxic for fish at concentrations higher than 1.5 mg NH_4^+-N·L^{-1}, this compound must be removed by a nitrifying biofilter to avoid its accumulation in the system. Ammonia is oxidized into nitrate which is less toxic for fishes, its recommended limit being around 50 mg NO_3^--N·L^{-1}. However its effect depends on the specie and growth stage and, therefore, its removal is advisable. The use of denitrifying biofilter with elemental sulphur would be the most suitable option to maintain nitrate concentration as low as possible (Figure 1.9). The sulphate generated during the autotrophic denitrification would cause neither environmental nor toxicity problems when marine species are cultured (Vidal *et al*., 2002).

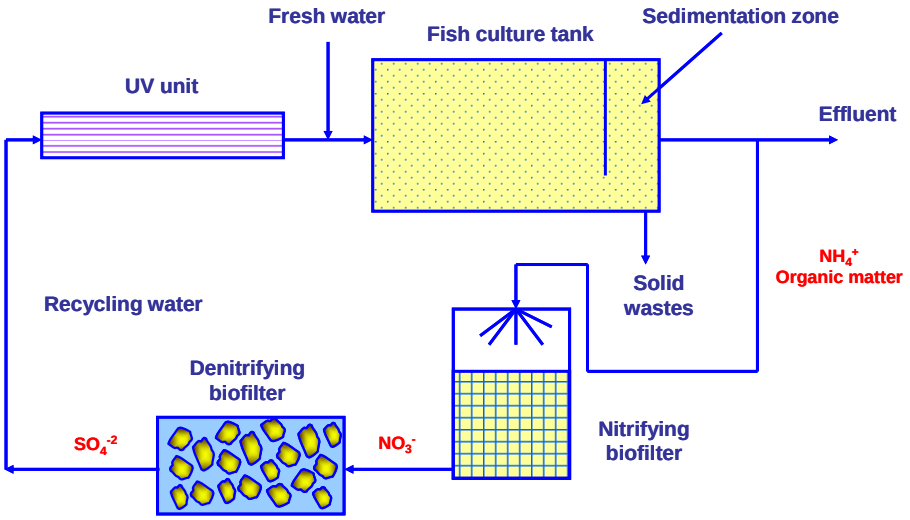

Figure 1.9 Recirculating aquaculture system.

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Chapter 2.

Materials and Methods

Summary

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

From the conventional chemical parameters measured in the liquid phase, the Total, Inorganic and Volatile Suspended Solids (TSS, ISS and VSS) were determined following Standard Methods (APHA-AWWA-WPCF, 2005). These are therefore not further described in this chapter. Nitrogen in the form of ammonium (NH_4^+), Total Organic and Inorganic Carbon (TOC, IC), sulphide and several inorganic anions (NO_2^-, NO_3^-, Cl^-; PO_4^{3-}, $S_2O_3^{2-}$ and SO_4^{2-}) have been measured by analytical procedures optimised in our laboratories and these are thus described in detail throughout this chapter. The biomass was characterised also by means of parameters such as TSS, ISS, VSS and SVI, and specific activity assays. Identification of the different populations present in the biomass samples was carried out by molecular techniques, Fluorescent In Situ Hybridization (FISH), PCR and Sequencing,

2.1. LIQUID PHASE

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

2.1.1. Ammonium nitrogen

Ammonium nitrogen was measured by means of a selective electrode (CRISON 96 63) together with a reference electrode of lithium acetate 0.1 M (CRISON 50 44), connected to a pH/mV meter (CRISON GLP-22) with a sensibility of 0.1 mV (Figure 2.1).

2.1.1.1. Reagents preparation

- Solution of lithium acetate 0.1 M (CRISON 99 01).
- Ionic strength adjuster (ISA), $MgSO_4$ 1 M (12.05 g $MgSO_4$ in 100 mL of deionised water).
- Ammonium stock standard solution contained 1.0 g $NH_4^+ \cdot L^{-1}$ (2.965 g of NH_4Cl, previously dried at 120 ºC for two hours, in 1000 mL deionised water).

2.1.1.2. Determination procedure

Firstly, the electrodes were calibrated by using a series of known standard solutions done by serial dilution of the stock standard solution (2 – 300 mg $NH_4^+ \cdot L^{-1}$). The electrode signal (mV) was obtained for each standard solution by adding 0.25 mL of ISA to 25 mL of standard/sample under well-stirred conditions.

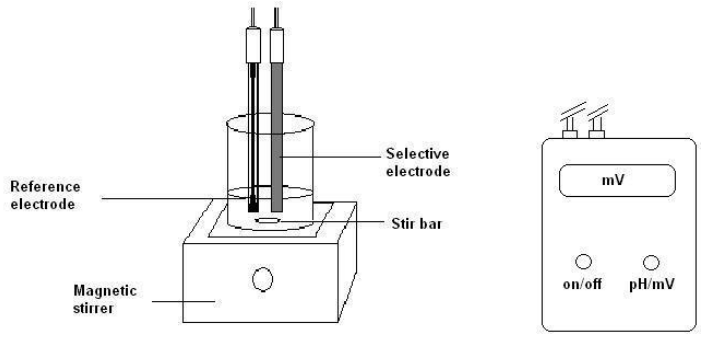

Figure 2.1 Equipment for ammonium analysis.

The calibration curve was obtained by plotting the electrode signal (mV) versus the logarithm of the ammonium concentration (Figure 2.2).

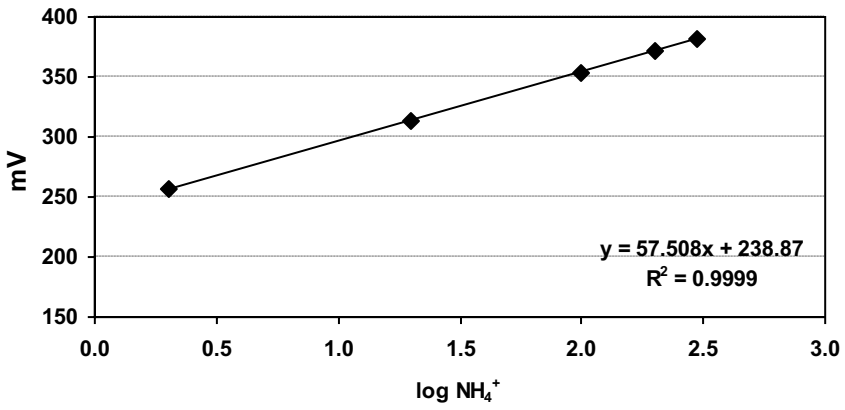

Figure 2.2 Calibration curve for ammonium concentration determination.

2.1.2. Analysis of inorganic anions: NO_2^-, NO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, and Cl^-

Nitrite, nitrate, sulphate, thiosulphate and chloride were determined by ion chromatography (Metrohm 861) equipped with a suppressed conductivity detector, sample

processor (Metrohm 838) and a Metrosep A column (250 x 4.0 mm). The mobile phase was a buffer with 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ at a flow rate of 0.7 mL·min^{-1}, 20 µL injection volume. Data collection was done by using the Processor software IC Net 2.3 (2006) Herisau, Switzerland.

2.1.2.1. Reagents preparation

- Solution of Na_2CO_3: 3.2 mM (339.2 mg Na_2CO_3 in 1000 mL deionised water).
- Solution of $NaHCO_3$: 1.0 mM (84 mg $NaHCO_3$ in 1000 mL deionised water).
- Standard stock solutions: nitrate (Fluka 74246), nitrite (Fluka 67276), sulphate (Fluka 90071), thiosulphate (Sigma S-1648, 1.4 g in 1000 mL deionised water) and chloride (Fluka 39883).

2.1.2.2. Determination procedure

Table 2.1 indicates the calibration concentrations ranges for the different inorganic anions. The calibration of each anion was done by serial dilutions of the corresponding stock standard solution (Figure 2.3). The samples were analyzed by triplicate.

Table 2.1 Concentrations ranges for the different inorganic anions (mg L^{-1}) to obtain the calibration curves.

Ion	Low value	High value
Cl^-	1.0	100
NO_2^-	0.5	5.0
NO_3^-	0.5	50
PO_4^{3-}	0.5	50
SO_4^{2-}	1.5	150
$S_2O_3^{2-}$	1.5	150

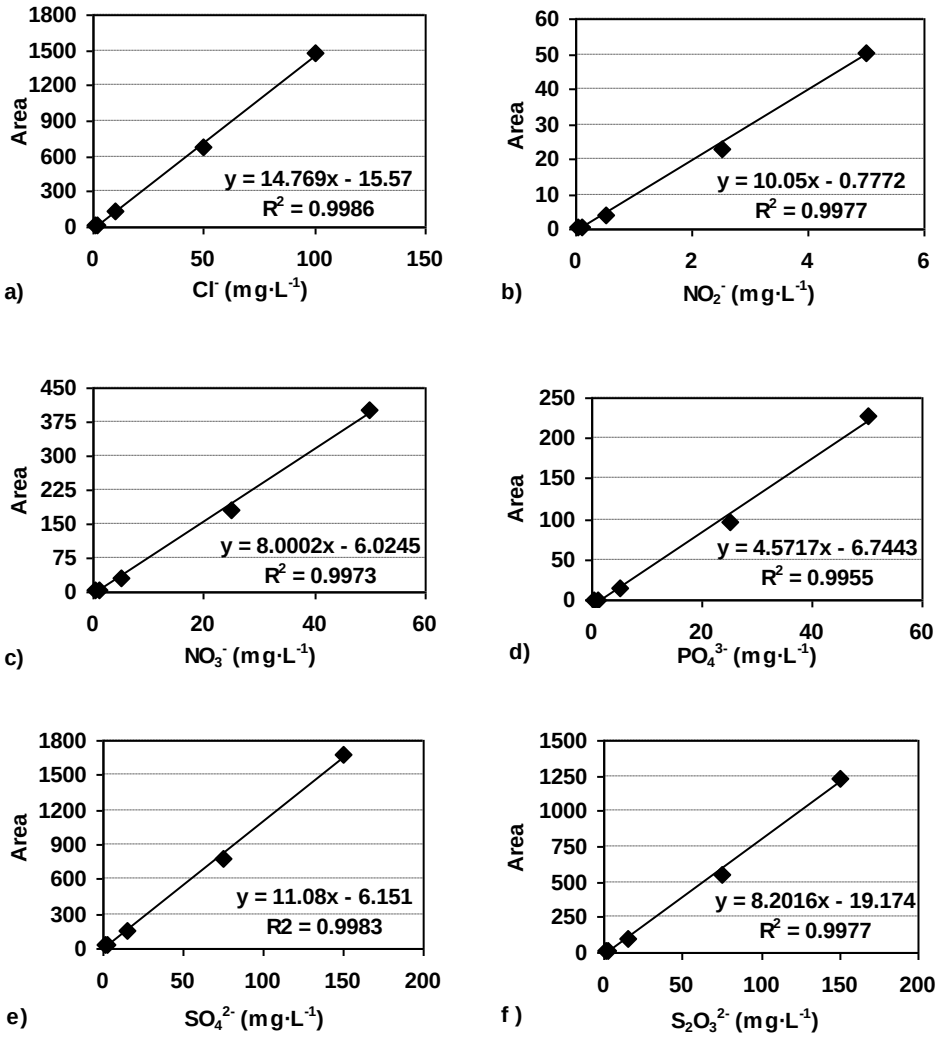

Figure 2.3 Calibration curves for: a) Cl^-, b) NO_2^-, c) NO_3^-,d) PO_4^{3-}, e) SO_4^{2-} and f) $S_2O_3^{2-}$.

2.1.3. Sulphide

Sulphide was measured by means of a selective electrode (Orion 9416BN) together with a double junction reference electrode (ORION 900200), connected to a pH/mV meter (CRISON GLP-22) with a sensibility 0.1 mV (Baldo *et al.,* 2002).

2.1.3.1. Reagents preparation

- Inner chamber solution (ORION 900002).
- Outer chamber solution (ORION 900003).
- Lead perchlorate solution 0.1 M (46 g $Pb(ClO_4)_2 \cdot 3\ H_2O$ in 1000 mL distilled water).
- Sulphide anti-oxidant buffer (SAOB) (80 g NaOH, 320 g sodium salicylate and 72 g ascorbic acid in 1000 mL distilled water. This solution must be prepared weekly).
- Stock solution of saturated sodium sulphide (approximately 100 g of reagent-grade $Na_2S \cdot 3H_2O$ in 100 mL distilled and deaerated water).
- Sulphide standard solution (1.0 mL of stock solution of saturated sodium sulphide, 50 mL of SAOB solution and 49 mL of distilled and deaerated water. This solution must be prepared weekly).

2.1.3.2. Determination procedure

The reference electrode had two chambers; the inner chamber was filled with solution Orion 900002 and the outer chamber with solution Orion 90003. Then the reference electrode was conditioned by soaking it consecutively in two sodium sulphide solutions (2% and 10%) for 5 minutes, rinsing it with distilled water and cleaning it with a soft cloth. Finally, the reference electrode was connected to the pH/mV meter.

To check the electrode performance, the response of the electrode was previously measured as follows:

Under well-stirred conditions, 50 mL of distilled water plus 50 mL of SAOB solution were placed in a 150 mL beaker. Then 1 mL of sulphide standard solution was added and

the electrode signal (mV) was recorded. Later 10 mL of sulphide standard solution was added to the beaker and a new signal was recorded. The difference between both signals was defined as the slope of the electrode and should be in the range of 25-30 mV.

2.1.3.2.1. Titration of sulphide standard solution

To determine the concentration of the sulphide standard solution was necessary in order to obtain the calibration curve. For this purpose, 25 mL of sulphide standard solution were titrated with a lead perchlorate solution (0.1 M) (Figure 2.4). The concentration of the sulphide standard solution was determined according to the following equation:

$$C = 3206\ (Vt / Vs) \qquad [2.1]$$

Where:

- C = S^{2-} concentration (mg L^{-1})
- V_t = Volume of lead perchlorate solution to reach the inflection point (mL)
- V_s = Volume of the sulphide standard solution (mL)

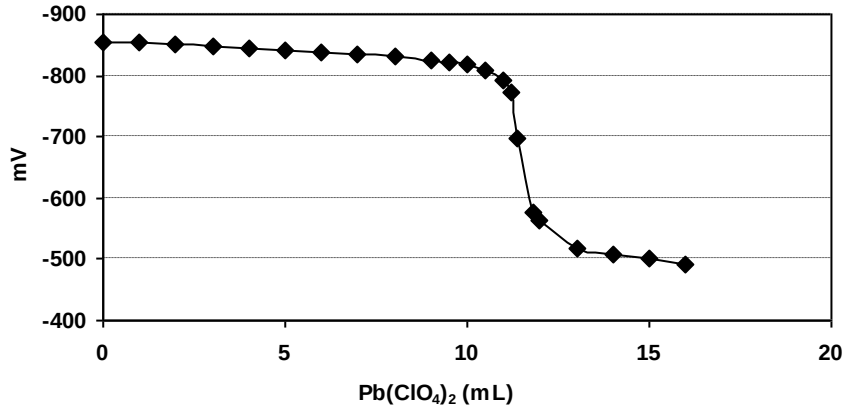

Figure 2.4 Titration curve of sulphide standard solution.

2.1.3.2.2. Calibration curve

Electrodes were calibrated by using solutions of known concentrations prepared by serial dilution of the sulphide standard solution. To obtain the different values of the calibration curve, the following procedure was done for each diluted solution: 10 mL of the solution were added to 45 mL of SAOB solution and 45 mL of distilled water. The solution obtained was stirred and the electrode signal (mV) was recorded. The calibration curve was obtained by plotting the electrode signal versus the logarithm of the S^{2-} concentration (Figure 2.5). This calibration curve was valid for only one week.

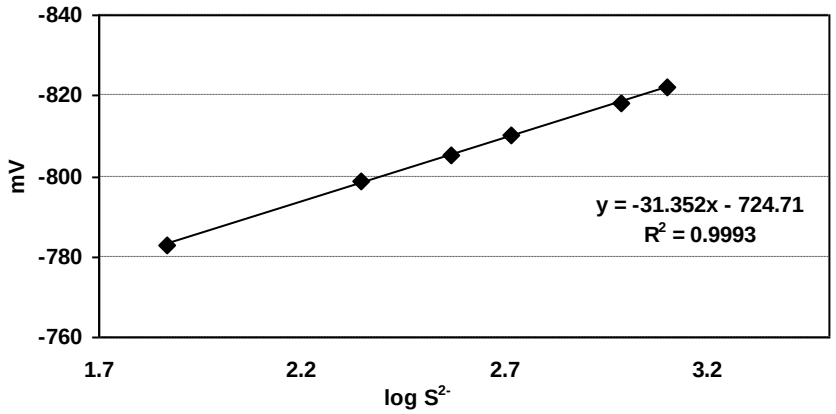

Figure 2.5 Sulphide calibration curve.

2.1.3.2.3. Sample analysis

A volume of 10 mL of sample were added to 45 mL of SAOB solution and 45 mL of distilled water. This solution was stirred and the electrode signal (mV) was recorded. Samples could be stored during one week in the SAOB solution before their analysis.

2.1.4. pH

The pH measurements were performed with an electrode (CRISON Instruments, 52-03) connected to a measure instrument (pH/mV) 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. GAS COMPOSITION

The biogas composition was analyzed by a Gas Chromatograph HP-5890 Series II with a Porapack Q 80/100 of 2 m x 1/8'' column (Supelco) and a conductivity detector. The temperature of both injector and detector was 110 ºC. The oven temperature was fixed at 35 ºC during 45 minutes, then, it was increased up to 100 ºC at a rate of 70 ºC/min and, finally it was fixed at 100 ºC for 3 minutes. The carrier gas was helium at a flow rate of 46 mL/min. The volume of the biogas sample injected was 1 mL.

2.3. BIOMASS CHARACTERIZATION

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

2.3.1. Total and Suspended Solids

Total Suspended Solids (TSS) Volatile Suspended Solids (VSS) and inorganic suspended solids concentrations were determined according to the methods 2540D and 2540E described in Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 2005).

2.3.1.1. Determination procedure

For the determination of the TSS concentration, a selected (in order to yield a residue between 2.5 and 200 mg) well-mixed sample volume was filtered through a previously 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 (2 h) at 103-105 ºC. The increase in weight of the filter represented the TSS concentration.

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

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

2.3.2. Sludge Volumetric Index

The Sludge Volumetric Index (SVI) determination is defined in the Standard Methods for the Treatment of Water and Wastewater (APHA-AWWA-WPCF, 2005) as the volume in millilitres occupied by 1 g of a suspension after 30 min of settling.

2.3.3. Specific Autotrophic Denitrifying Activity (ADA) and sulphur oxidant activity (SOx) measurement

Two kinds of experiments were performed to estimate the activity of the autotrophic denitrifying biomass: a) those performed directly in the reactor by monitoring the liquid phase (LPR) and b) those performed in vials with biomass samples collected from the reactor by monitoring either the liquid phase (LPV) or the gas phase (GPV).

2.3.3.1. Experiments performed in the reactor (LPR)

The specific ADA was measured by monitoring the sulphur and nitrogen compounds concentrations in the liquid phase (LP) of the reactor. Initially the biomass was allowed to settle during around 30 min, one litre of supernatant was removed and replaced by the same volume of phosphate buffer solution (0.143 g $KH_2PO_4 \cdot L^{-1}$ and 0.747 g $K_2HPO_4 \cdot L^{-1}$, pH fixed at 7.45) to remove the possible amounts of nitrate or thiosulphate present in the liquid medium. This procedure was repeated three times. Then 100 mL of buffer containing the substrates were added to the reactor to keep the initial concentrations at 300 mg $S_2O_3^{2-}$-$S \cdot L^{-1}$ and 100 mg NO_3^--$N \cdot L^{-1}$. Temperature was maintained at 30 ºC by means of the thermostatic jacket. Liquid samples from the supernatant were collected every hour, filtrated through Nylon membranes of 0.45 µm and analyzed by ion chromatography. The maximum ADA was estimated from the maximum slope of the curves described by the concentrations of nitrate and thiosulphate consumption along the time and the biomass concentration in the reactor.

2.3.3.2. Experiments performed in vials (LPV and GPV)

The assays in liquid phase (LPV) and gas phase (GPV) 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 useful volume were used. Biomass concentration at the beginning of the experiment was fixed around 1.0 g VSS·L^{-1}. Before the beginning of the batch test the biomass was washed three times with phosphate buffer (0.143 g KH_2PO_4·L^{-1} and 0.747 g K_2HPO_4·L^{-1}). The pH value was fixed at 7.5 and the temperature was fixed at a certain value T depending on the conditions to be analyzed. Gas and liquid phases were purged with He gas to remove O_2. The vials were placed in a thermostatic shaker, at 150 rpm and the temperature T, until stable conditions were reached.

Initial concentrations of substrates were adjusted according to the test to be analyzed.

For the analysis of the liquid phase, samples from the supernatant were collected at different intervals of time, and filtrated in Nylon membrane of 0.45 µm, the composition was analyzed by Ion chromatography (Section 2.1.2) and selective electrode (Section 2.1.3). For the assays GPV the biogas production in the headspace of the vials was measured by means of a pressure transducer, and biogas composition was analyzed by gas chromatography (Section 2.2).

The maximum specific ADA was estimated from the maximum slope of the curve described by substrates consumed (LPV) and the cumulative N_2 production (GPV) along the time and related to the biomass concentration in the vials. SOx activity was estimated from the maximum slope of the curve described by substrates consumed for those cases when liquid phase was measured, our according to stoichiometric ratio.

The N_2 gas production rate (moles N_2·min^{-1}) was calculated from the maximum slope of the curve describing the pressure increase in the vial along time (α) (atm·min^{-1}) (Eq. 2.2).

$$\frac{dN_2}{dT} = \alpha \frac{V_g}{R \cdot T} \qquad [2.2]$$

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

The maximum specific ADA (g N_2-N·g VSS^{-1}·d^{-1}) was calculated from the N_2 gas production rate and the biomass concentration in the vial (g VSS·L^{-1}):

$$\text{ADA} = \frac{dN_2/dT}{X \cdot V_L} \cdot \frac{28\,g\,N}{mol\,N_2} \cdot \frac{1440\ \text{min}}{d} \qquad [2.3]$$

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

2.3.4. Specific nitrifying activity measurement

Nitrifying 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 concentrations along the 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. This system is a discontinuous respirometer that uses 15 mL vials with a maximum useful volume of 10 mL (Figure 2.6).

The biomass was washed with phosphate buffer (1.43 g·L^{-1} KH_2PO_4, 7.47 g·L^{-1} K_2HPO_4). A volume of 10 mL of biomass suspended in the buffer medium was added to each vial. Vials were placed in a thermostatically controlled chamber at 25 ºC. Compressed air was used to obtain the oxygen saturation in the liquid medium. The two electrodes for oxygen measurement were calibrated. Then, the electrodes were carefully inserted into the vials in order to avoid the presence of bubbles in the liquid surface, 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 steep slope. After 10 minutes, the test was finished and the biomass concentration in each vial was measured as VSS. With the measured oxygen consumption rate, the specific activity was calculated as the substrate concentration consumption rate divided by

the biomass concentration as g $O_2 \cdot L^{-1} \cdot d^{-1}$.

Figure 2.6 Respirometric system: left, thermostated test cells; center, biological oxygen monitor; right, data acquisition system.

2.3.5. Microbiological determinations

2.3.5.1. Phylogenetic analysis

2.3.5.1.1. DNA extraction

Total community DNA was extracted from biomass samples following a bead beating protocol using a PowerSoil DNA soil extraction kit (MoBio Laboratories, Inc., Solano Beach, CA) following the manufacturer´s instructions.

The 16S rRNA gene hypervariable regions of bacteria V3-V5 were amplified using primers 16F341-GC (5´-CCTACGGGAGGCAGCAG-3´) and 16R907 (5´-CCGTCAATTCCTTTRAGTTT-3´) (Yu and Morrison, 2004). Primer F341-GC included a GC clamp at the 5´end (5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3').

2.3.5.1.2. PCR-DGGE

Genomic DNA was subjected to DGGE analysis as previously described (Alonso-Gutierrez *et. al*., 2009). PCRs were performed using Veriti Thermocycler (PE Applied Biosystems, Foster City, California, USA) in a volume of 50 µL containing 1.25 U of Taq (TaKaRa ExTaq Hot Start Version; TaKaRa Bio Inc., Otsu, Siga, Japan), 1X ExTaq Buffer (2 mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 0.5 µM of primers and 100 ng of template DNA. After 9 min of initial denaturation at 95 °C, a touchdown thermal profile protocol was carried out, and the annealing temperature was decreased by 1 °C per cycle from 65 °C to 55 °C; followed by 25 additional cycles of 1 min of denaturation at 94 °C, 1 min of primer annealing at 55 ºC, and 1.5 min of primer extension at 72 °C, followed by 10 min of final primer extension at 72 ºC.

Approximately 800 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel that was 0.75 mm thick with denaturing gradients and ranged from 40 to 75% denaturant concentrations (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1X TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA; pH 8.4) using an INGENY PhorU system (Ingeny, Goes, The Netherlands) at 100 V and 60 °C for 17 h. DGGE gels were stained with 1X TAE buffer containing SybrGold (Molecular Probes, Inc., Eugene, OR, USA). Predominant DGGE bands were excised with a sterile razor blade, suspended in 50 µl sterilized MilliQ water, stored at 4 °C overnight, reamplified by PCR using primers F341-R907 and sequenced as described below.

2.3.5.1.3. Sequencing

In order to eliminate the excess of primers and dNTPs for sequencing reactions, the PCR products were digested at 37 °C for 1 hour using Shrimp Alkaline Phosphatase (SAP) ($1U \cdot \mu L^{-1}$) and Exonuclease I (ExoI) ($10U \cdot \mu L^{-1}$) (U.S.B. Corporation, Cleveland, OH, USA). The enzymes were afterwards inactivated by heating the samples at 80 °C for 15 min.

Sequencing was accomplished using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) and an ABI PRISM 3700 automated sequencer (PE Applied Biosystems, Foster City, California, USA) following the manufacturer´s instructions.

2.3.5.1.4. Phylogenetic analysis

Phylogenetic analysis was performed as previously described (Alonso-Gutierrez *et al*., 2009b) with some modifications. Sequences were inspected, corrected and assembled into a single consensus sequence for each phylotype. After that, the sequences were examined with the BLAST search alignment tool comparison software (BLASTN) (Altschul *et al*. 1990) to detect the closest prokaryotic group to each sequence among GenBank database. Sequences from all phylotypes were aligned with reference sequences obtained from GenBank using MAFFT (Katoh *et al*., 2005). The alignment obtained was transferred to MacClade (Maddison & Maddison, 2003) and finally edited using the MacClade program. The edited alignments were directly transferred to jMODELTEST software version 3.6 (Posada & Crandall, 1998) as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment. jMODELTEST examines ML models, ranging from simple to complex. We allowed for rate variation across sites, assuming a gamma distribution (0.4890) and a proportion of invariable sites (0.2580) estimated by using jMODELTEST (Akaike information criterion; Posada & Crandall, 1998Go). Base frequencies as determined by using jMODELTEST for A, C, G and T were 0.2759, 0.1844, 0.2797 and 0.2600, respectively, with the rate matrix of the substitution model being 1.0000 (AC), 1.8124 (AG), 1.0000 (AT), 1.0000 (CG), 2.9845 (CT) and 1.0000 (GT). We assessed the relative stability of the tree topology by using 1000 distance bootstrap replicates and 100 maximum-likelihood bootstrap replicates. The settings for bootstrap calculations were the same as those given above. These best-fit models of nucleotide evolution, calculated by jMODELTEST were incorporated into software PHYML (Guidon & Gascuel, 2003), which uses a single, fast, and accurate algorithm to estimate large phylogenies by ML. Finally, the trees created by PHYML were edited using the FIGTREE v1.1.2 software (http://tree.bio.ed.ac.uk/software/figtree/).

2.3.5.2. Identification of bacterial populations by FISH technique

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

According to Amann *et al*. (1995) a typical FISH protocol includes four steps (Fig. 2.7): the fixation and permeabilization of the sample; hybridization of the targeted

sequence to the probe; washing steps to remove unbound probe; and the detection of labelled cells by microscopy or flow cytometry. This protocol must be applied to disrupted biomass; therefore, the granules must be disintegrated before starting the procedure. To achieve the granular biomass breakage, biomass was sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher).

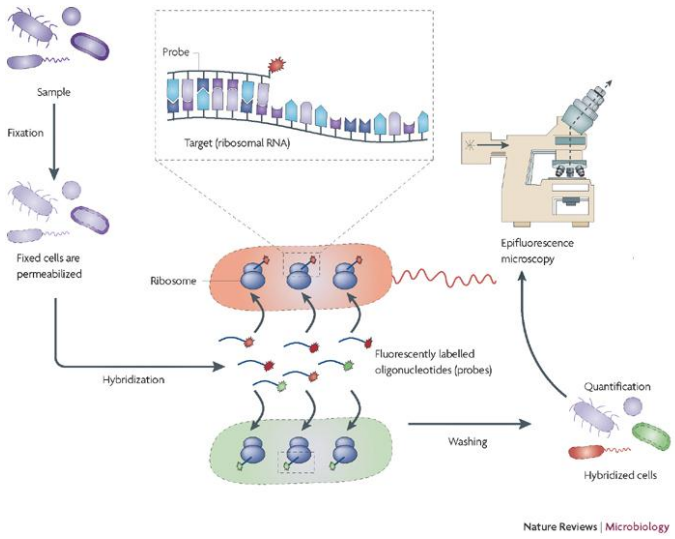

Figure 2.7 Basic steps of FISH technique. Adapted from Amann and Fuchs, 2008.

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

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

The fluorochromes used to detect the hybridized rRNA were FLUOS (5(6) carboxyfluorescein-Nhydroxysuccinimide ester) and Cy3 (indocarbocyanine). To visualize

all cells in a sample the stain 4,6- diamidino-2-phenylindole (DAPI) was used. Its application can provide insight into the existence of archaeobacteria and eukaryotes, like e.g. protozoa. For analysis of the slides an epifluorescence microscope (Axioskop 2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics) were used. Quantification of the bacterial population was based on the procedure published by Crocetti *et al.* (2002). The quantification was performed by comparison of the positive area obtained with a probe with the area corresponding to the control, DAPI. The digital image analysis software Image ProPlus® was used to quantify the areas.

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Chapter 3.

Enrichment of Autotrophic Denitrifying Biomass in a Feed Batch Reactor

Summary

In this chapter a batch reactor was used for the enrichment of autotrophic denitrifying biomass using thiosulphate as electron donor.

The obtained results demonstrated that it was possible to obtain an autotrophic denitrifying biomass able to use different sulphur sources as electron donor. The maximum specific autotrophic denitrifying activity (ADA) measured using thiosulphate as electron donor was of 211.6 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} while the obtained ADA in the presence of sulphide as electron donor was lower than the previous one of 153 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}.

The presence of sulphur stored compounds in the sludge was confirmed by the fact that a value of the ADA of 121 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} was measured in batch assays performed with enriched biomass in the absence of added sulphur source or another electron donor.

3.1 INTRODUCTION

Many industries generate wastewaters with high concentrations of nitrogen and sulphur compounds which contribute to the imbalance of both elements natural cycles. Anthropogenic sulphur and nitrogen compounds cause severe effects on the environment. Discharges of wastewater with high sulphate and nitrate concentrations can cause in the receiving bodies problems such as eutrophication. As an example the petrochemical industry produces wastewater with high concentrations of reduced sulphur compounds like hydrogen sulphide, which is toxic, corrosive and produces bad odours. The combustion of sulphide produces sulphur dioxide (SO_2) which contributes to the acid rain. Furthermore the sulphide causes serious human health problems even at very low concentrations: mucous membranes irritation and headache, dizziness, nausea and unconsciousness (Syed *et al.,* 2006; van den Bosch *et al*., 2007).

In nature there are a number of micro-organisms that are able to perform processes combining the nitrogen and sulphur cycles. The coupling of both cycles allows the simultaneous oxidation of reduced forms of sulphur and the reduction of nitrate or nitrite. This is a biological process known as autotrophic denitrification (Robertson and Kuenen, 1992; Fdz-Polanco *et al*., 2001).

Autotrophic denitrification with sulphur compounds such as SO_3^{2-}, $S_2O_3^{2-}$ and H_2S has been widely studied as an alternative for the post-treatment of wastewater, groundwater or drinking water (Moon *et al*., 2004, 2006; Soares, 2002). In this biological process the carbon source is provided by an inorganic compound and the nitrogen oxides are used as electron acceptor for the oxidation of sulphur compounds while N_2, SO_4^{2-} and Sº are the final products (Kleerebezem and Méndez, 2002; Fernández *et al*., 2008). These compounds are innocuous to the environment.

In nature thiosulphate plays an important role in the sulphur cycle and is an easily biodegradable compound compared to elemental sulphur and less toxic that sulphide. In previous studies nitrogen degradation rates with thiosulphate have been reported to be 4.6 and 9.5 folds higher than those obtained with sulphide and elemental sulphur, respectively (Beristain *et al.,* 2006). For this reason thiosulphate is the more widely used substrate for the enrichment of autotrophic denitrifying biomass (Wang *et al*., 2005; Moon *et al.,* 2004; Koenig *et al.,* 2005; Soares, 2002). However, there is few information regarding the main metabolic properties (Beristain *et al.,* 2006; Krishnakumar and Manilal, 1999; Oh *et al.,* 2000), populations involved (Moon *et al.,* 2004; Fernández *et al*., 2006, 2008, Wang *et al.,*

2005) and physicochemical properties of the sludge generated during the enrichment period.

In the last years the application of the autotrophic denitrification process to the wastewater treatment is being under study. It has been observed that the start up of the autotrophic reactors is the bottleneck of the process due to the lack of accurate inoculum sources. Either anaerobic or activated sludge are used as inoculum to develop denitrifying autotrophic biomass. In these cases long start up periods are required due to the slow growth rates of these micro-organisms of 0.11 - 0.20 h^{-1} (Claus and Kutzner, 1985). Enrichment of this kind of biomass has been achieved using as inoculum tidal flat (Kim and Bae, 2000; Moon *et al*., 2004; Koenig and Liu, 2001; Gu *et al*., 2004; Soares, 2002), activated sludge (Lau *et al*., 2006; Nugroho *et al.,* 2002) and anaerobic sludge (Oh *et al*., 2000; Krishnakumar and Manilal, 1999; Beristain *et al*., 2006; Sierra-Alvarez *et al*., 2007).

3.2 OBJECTIVES

The objective of this work was to study the development of an autotrophic denitrifying inoculum using sulphur compounds as electron donors and to monitor the specific autotrophic denitrifying activity of the biomass.

3.3 MATERIALS AND METHODS

3.3.1 Reactor

This work has been carried out in a stirred tank glass reactor with a total volume of 5 L and a working volume of 1.5 L. Dimensions of the unit were, height of 0.6 m and inner diameter of 0.12 m. The reactor mixture was achieved by mechanical stirring at 150 rpm and the temperature was controlled at 30 ºC by means of a thermostatic jacket (Figure 3.1).

Figure 3.1 Picture of the enrichment reactor.

3.3.2 Inoculum

The reactor was inoculated with 450 mL of anaerobic sludge from a wastewater treatment plant treating the effluents from a fish canning industry containing 52.6 g TSS·L^{-1}, 19.3 g VSS·L^{-1} and characterized by a sludge volumetric index (SVI) of 31 mL·(g VSS)$^{-1}$.

3.3.3 Feeding composition

The feeding medium was prepared according to Baldensperger and García (1975) by mixing a mineral medium with a micronutrients solution (Tables 3.1 and 3.2). Sulphur was added in the form of thiosulphate.

Table 3.1 Mineral medium composition

Compound	$g \cdot L^{-1}$
$Na_2S_2O_3 \cdot 5\ H_2O$	4.2 - 5.8
$NaNO_3$	1.2
$NaHCO_3$	1.5
Na_2HPO_4	1.5
KH_2PO_4	0.3
NH_4Cl	0.1
Micronutrients solution	1 $mL \cdot L^{-1}$

Table 3.2 Micronutrients solution composition

Compound	$g \cdot L^{-1}$	Compound	$g \cdot L^{-1}$
$Na_2MoO_4 \cdot 7H_2O$	1.0	$CuSO_4 \cdot 5H_2O$	0.25
$FeSO_4 \cdot 7H2O$	30.0	$CoCl_2 \cdot 6H_2O$	0.25
$ZnCl_2 \cdot 4H_2O$	1.0	HCl (32%)	50.0
$CaCO_3$	2.0	$NiCl_2 \cdot 6H_2O$	0.25
$MnCl_2 \cdot 4H_2O$	1.5	H_2BO_3	0.50

3.3.4 Operational strategy

The reactor was operated in batch mode in consecutive operational cycles during 95 days. Cycles comprised the influent addition (5 minutes), the mixed reaction phase (several days), the settling phase (30 minutes) and the effluent removal (5 minutes). The first three cycles ended on days 6, 12 and 19 of operation and from day 32 on the sequential feeding was performed each 3 days. The hydraulic retention time (HRT) varied from 4.5 to 10.5 days. Each cycle a volume of 1000 mL of reactor medium was substituted by the same volume of feeding medium. Removal of the effluent and feeding addition were performed manually from the top of the reactor.

3.3.5 Batch specific autotrophic denitrifying activity (ADA) and sulphur oxidizing activity (SOA) experiments

Two kinds of experiments were performed to estimate the activity of the autotrophic denitrifying biomass: a) those performed directly in the reactor by monitoring the liquid phase (LPR) and b) those performed in vials with biomass samples collected from the reactor by monitoring either the liquid phase (LPV) or the gas phase (GPV) according to the methodology described in chapter 2 and following the experimental design described in

the Table 3.3. All assays were performed at 30 ºC and 150 rpm. The initial biomass concentration in all experiments was fixed at 1.0 g VSS·L^{-1}.

Table 3.3 Batch specific ADA experiments to evaluate the ability of the enriched sludge to use different electron donors

Assay	Nitrogen compound (mg NO_3^--N·L^{-1})	Sulphur compound (mg S·L^{-1})	S/NO_3^- (mol·mol^{-1})	S source
1	100	300	1.3	$S_2O_3^{2-}$S
2	45	16	0.15	S^{2-}
3	22	32	0.63	S^{2-}
4	28	45	0.7	S^{2-}
5	80	440	2.4	S^0

The stoichiometric S/NO_3^- ratios corresponding to the autotrophic denitrification process with different sulphur sources are: 1.25 mol·mol^{-1} for thiosulphate, 0.625 mol·mol^{-1} for sulphide and 0.83 mol·mol^{-1} for elemental sulphur.

3.3.6 Analytical methods

The pH, volatile suspended solids (VSS) and total suspended solids (TSS) concentrations and sludge volumetric index (SVI) values were determined according to the Standard Methods (APHA, 2005).

Nitrite, nitrate, thiosulphate and sulphate concentrations were determined by ion chromatography, sulphide concentration was measured by means of an ion-selective electrode and the biogas composition by gas chromatography (see chapter 2 for detailed information).

3.4 RESULTS

3.4.1 Enrichment period

During the enrichment period the sludge was fed with nitrate and thiosulphate at concentrations of 0.198 g NO_3^--N·L^{-1} and 1.08-1.50 g $S_2O_3^{2-}$-S·L^{-1}. Under these conditions the S/NO_3^- ratio was of 2.4-3.3 mol·mol^{-1} (operation under nitrate limiting conditions) and the specific NLR was of 1.36 ± 0.22 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}.

In each of the three initial operational cycles the two thirds of the total reactor volume were extracted at a frequency of 6-7 days and replaced by the same amount of feeding media. During these three first feedings nitrate was removed from the liquid media in approximately six days and a denitrification rate of 49 mg NO_3^--N·L^{-1}·d^{-1} was estimated from the maximum slope of the curve describing the nitrate concentration along time. This value is higher than that reported by Manconi *et al.* (2007) who operated a fed-batch reactor for the enrichment of *Thiobacillus denitrificans* cells immobilized into the flocs of activated sludge. In the case of the fourth feeding (on day 19 of operation) the denitrification rate improved significantly up to a value of 148 mg NO_3^--N·L^{-1}·d^{-1}. This value is slightly higher than those previously reported by Beristain *et al*. (2006) who operated an UASB reactor for the enrichment of autotrophic biomass with thiosulphate as electron donor. They tested in batch assays the activity of the biomass grown on three sulphur sources and found that the denitrification rate with thiosulphate was five and ten folds higher that those obtained with sulphide and elemental sulphur, respectively. In the case of the subsequent feedings, to fit the sulphur mass balances performed to the reactor was not possible and periodical activities assays were performed in the reactor to determine the evolution of nitrogen and sulphur compounds.

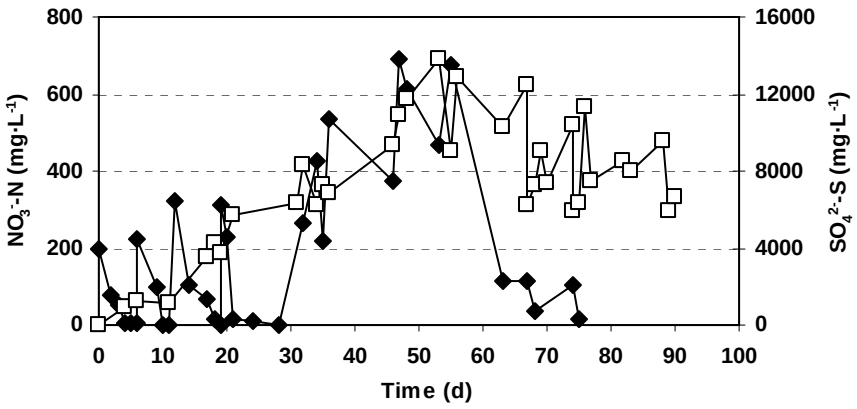

Figure 3.2 Concentrations of NO_3^--N consumed (♦) and SO_4^{2-}-S produced (□) in the reactor.

Sulphate production was detected in the liquid media from beginning of the reactor operation indicating that the sludge used as inoculum contained some micro-organisms

able to oxidize thiosulphate into sulphate. The concentration of sulphate in the reactor was gradually increased to values up to 12 g SO_4^{2-}-S/L. This value did not correspond to the amount of thiosulphate added in each feeding (1.54 g $S_2O_3^{2-}$-S·L^{-1}) but it is a common behaviour of fed batch reactors, where accumulation of the product can be registered (Figure 3.2).

During the enrichment period a gradual change of suspended solids concentrations was observed. The inoculum contained 52.6 g TSS·L^{-1} and 19.3 g VSS·L^{-1} that corresponded to a VSS/TSS ratio of 0.37 g·g^{-1}. This value indicated the high concentration of inorganic compounds present in the sludge (Table 3.4). The evolution of the VSS concentration during the enrichment period indicated a significant loss of biomass with a reduction of its concentration from 19.3 g·L^{-1} to 14.2 g·L^{-1} at end of the fourth feeding (Figure 3.3). At this moment the selection of biomass able to carry out autotrophic denitrification was achieved (Figure 3.4). At end of operation the VSS concentration in the reactor decreased to more than half and registered a value of 7.8 g VSS·L^{-1} and with a VSS/TSS ratio of 0.55 g·g^{-1} (Table 3.4). This value agrees with that reported by Chen *et al*. (2008) who operated an EGSB reactor for simultaneous removal of nitrogen, carbon and sulphide. Despite this the remained biomass improved its specific ADA (Figure 3.4).

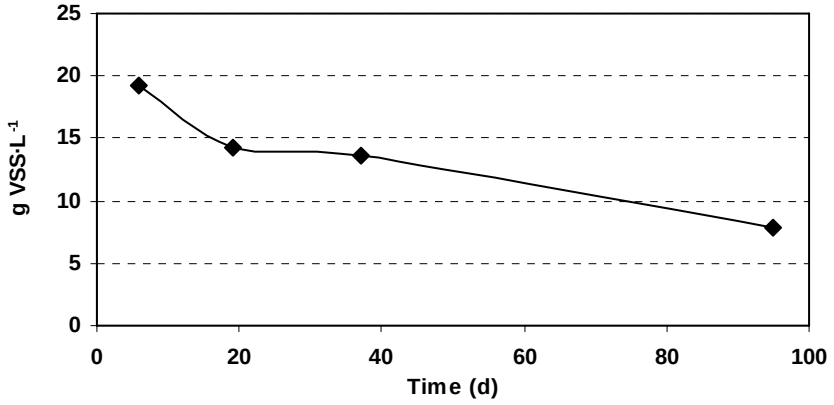

Figure 3.3 Evolution of the VSS concentration inside the reactor.

The physical properties of the sludge were maintained almost constant along the time. The initial SVI value of the inoculum was of 31 mL·(g VSS)$^{-1}$, which is a value in the range of those reported for anaerobic sludge from 13.95 to 82.5 mL·(g·VSS)$^{-1}$ (Moreno and

Buitrón 2002). At the end of the operation the sludge had an SVI of 33.3 mL·(g VSS)$^{-1}$ (Table 3.4).

Table 3.4 Evolution of the physical properties of the sludge during the enrichment period

	Inoculum	90 days
TSS (g·L^{-1})	52.6	14.2
VSS (g·L^{-1})	19.3	7.8
VSS/TSS ratio (g·g^{-1})	0.37	0.55
SVI (mL·(g VSS)$^{-1}$)	31.0	33.3

3.4.2 Autotrophic denitrifying activity assays

3.4.2.1 Experiments performed in the reactor (LPR)

To follow up the enrichment process of the biomass the autotrophic denitrifying activity in terms of nitrogen reduction and thiosulphate oxidation was estimated for each operational cycle from the evolution of the concentrations of these compounds measured along the time.

The biomass from the reactor experienced an increment of the specific autotrophic denitrifying and sulphur-oxidizing activities along the time (Figure 3.4). This indicated a progressive enrichment of the biomass on autotrophic denitrifying micro-organisms. Initial measured activities were of 2.4 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} and 9.5 mg $S_2O_3^{2-}$-S·(g VSS)$^{-1}$·d^{-1}, respectively. The autotrophic denitrifying activity was ten times lower than that previously reported by Manconi *et al.* (2007) for activated sludge. This behaviour could be due to the source of used inoculum in this work, which was an anaerobic sludge from anaerobic digester treating wastewater from fish canning industry where no significant sulphate reduction activity occurred. On day 32 of enrichment both activities increased up to 10 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} and 39.3 mg $S_2O_3^{2-}$-S·(g VSS)$^{-1}$·d^{-1}, respectively.

The specific ADA and SOA registered values were of 90 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} and of 187 mg $S_2O_3^{2-}$-S·(g VSS)$^{-1}$·d^{-1}, respectively, at the end of the operational period. Despite this improvement the values were lower that those reported in the literature for acclimated biomass (Beristain *et al*., 2006; Campos *et al.,* 2008) due to the different sources of inoculum used. Beristain *et al*. (2006) used as source of inoculum for the enrichment of autotrophic denitrifying biomass an anaerobic sludge from an UASB reactor treating the

effluents from a recycle paper factory and Campos *et al.* (2008) used autotrophic denitrifying biomass pre-enriched in an UASB reactor with nitrate and sulphide.

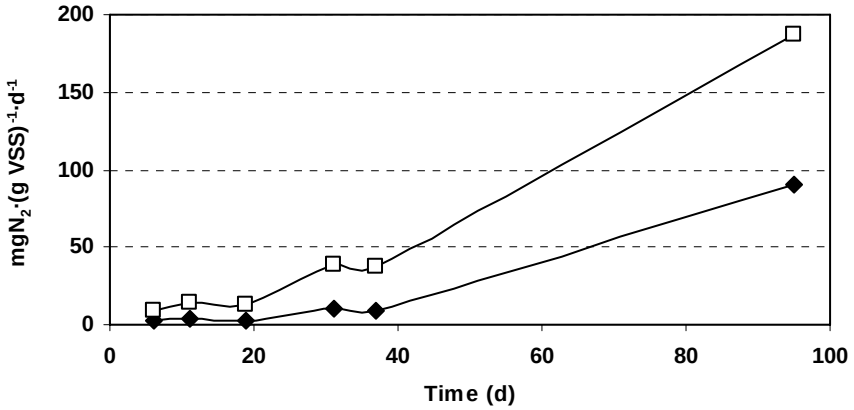

Figure 3.4 Evolution of the autotrophic denitrifying activity (ADA) (♦) and the sulphur-oxidizing activity (SOA) (□) during the enrichment period.

3.4.2.2 Experiments performed in vials (LPV and GPV)

At the end of the enrichment period the specific ADA and SOA values corresponding to the reactor biomass were measured using different reduced sulphur compound ($S_2O_3^{2-}$, S^{2-}, S^o) in batch assays following the liquid and gas phases (LPV and GPV).

3.4.2.2.1 Specific ADA in the presence of thiosulphate as electron donor (LPV)

The concentrations of substrates were fixed at 300 mg $S_2O_3^{2-}$-S·L^{-1} and 100 mg NO_3^--N·L^{-1} respectively, corresponding to a S/NO_3^- of 1.3 mol·mol^{-1} (close to stoichiometric conditions) (Assay 1; Table 3.3).

The initial and final concentrations of the compounds in the liquid phase corresponding to this assay are shown in Table 3.5. Thiosulphate was fully oxidized to sulphate while under these conditions only 51% of the fed NO_3^--N was recovered as nitrogen gas. Residual nitrate and nitrite were detected at the end of the assay (Table 3.5).

Table 3.5 Initial and final concentrations of substrates and products in the batch experiments performed with thiosulphate (S/NO_3^- of 1.3 mol·mol^{-1}) (Assay 1; Table 3.3)

NO_3^--N initial (mg·L^{-1})	$S_2O_3^{2-}$-S initial (mg·L^{-1})	NO_3^--N final (mg·L^{-1})	NO_2^--N final (mg·L^{-1})	N_2–N final (mg·L^{-1})	SO_4^{2-}-S final (mg·L^{-1})
100	300	15.5	33.0	47.7	255

The concentration profiles of the different compounds measured in the liquid phase and the nitrogen gas produced in the gas phase are shown in Figure 3.5. Thiosulphate was fully oxidized in 150 minutes while an important amount of nitrate was left. Nitrite slightly appeared after this moment with the simultaneous decrease of the nitrate concentration. These results agree with those reported by Campos *et al*. (2008) who performed batch assay at different S/N ratios. In those assays where thiosulphate was the limiting compound, they observed that nitrate was never depleted and nitrite was present at end of the assay which indicated the incomplete denitrification.

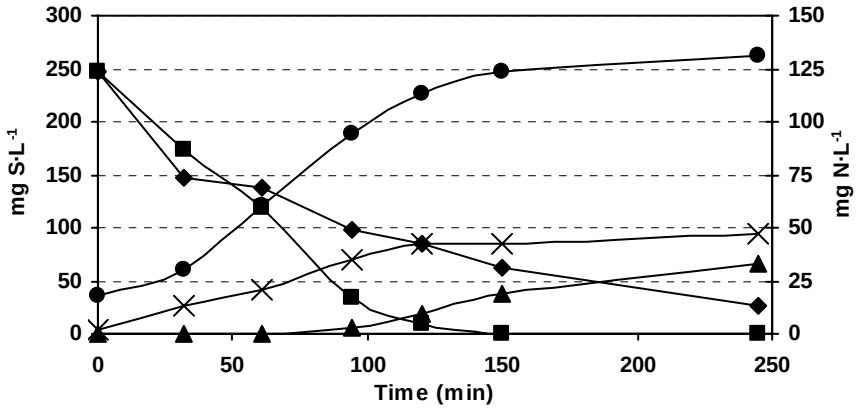

Figure 3.5 Evolution of nitrogen and sulphur compounds concentrations during the batch assay with thiosulphate. $S_2O_3^{2-}$-S (■), SO_4^{2-}-S (●) NO_3^--N (♦), NO_2^--N (▲) and N_2–N (**X**).

The values of the specific ADA and SOA estimated for this assay were 211 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} and 643 mg $S_2O_3^{2-}$-S (g VSS)$^{-1}$·d^{-1}, respectively. This value of the ADA is in accordance with Beristain *et al.* (2006) who tested different sulphur sources and found that the maximum denitrifying activity was with thiosulphate as electron donor of 211 mg NO_3^-

$N \cdot (gVSS)^{-1} \cdot d^{-1}$ for biomass acclimated to thiosulphate and nitrate in an UASB reactor fed with a liquid media prepared under stoichiometric conditions.

3.4.2.2.2 Specific ADA in the presence of sulphide as electron donor (LPV and GPV)

In order to determine if this sludge was capable of using sulphide as electron donor different assays were carried out at different S^{2-}/NO_3^- ratios. The theoretical value of the S/NO_3^- ratio for autotrophic denitrification with sulphide as electron donor is reported as 0.625 $mol \cdot mol^{-1}$. In the present work S/NO_3^- ratios of 0.15, 0.63 and 0.7 $mol \cdot mol^{-1}$ were tested (sulphide limiting, stoichiometric and nitrate limiting conditions, respectively) (Assays 2, 3 and 4; Table 3.3).

At an S/N ratio of 0.15 $mol \cdot mol^{-1}$ the nitrate was almost completely reduced to nitrogen gas in 100 minutes (Figure 3.6). Nitrite was detected as intermediate of the denitrification and it was slowly reduced to nitrogen gas until complete depletion after 150 minutes. Gommers *et al*. (1988) studied the simultaneous sulphide and acetate oxidation in a denitrifying fluidized bed reactor and obtained similar results with a fast sulphide consumption and a slow nitrate reduction with nitrite accumulation. They attributed this behaviour to differences between nitrate and nitrite reduction rates. Gadekar *et al*. (2006) found that under stoichiometric conditions and using sulphide as sulphur source nitrite was accumulated at a rate of 5.46 mg $N \cdot h^{-1}$ while the value for nitrite reduction rate was of 4.62 mg $N \cdot h^{-1}$.

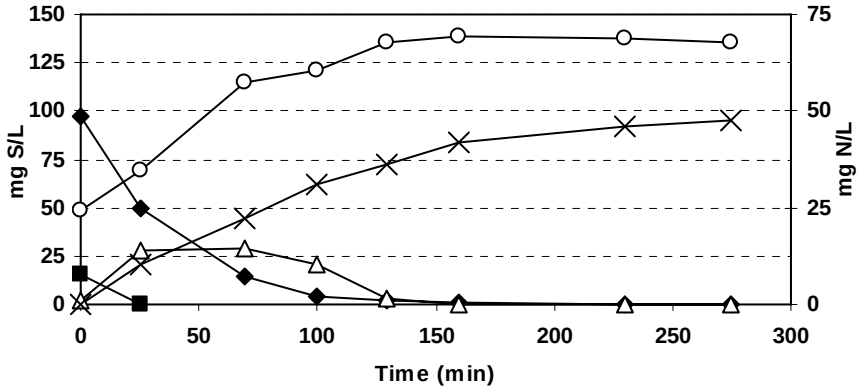

Figure 3.6 Evolution of nitrogen and sulphur compounds concentrations during the batch assay with sulphide at a S/NO_3^- ratio of 0.15 $mol \cdot mol^{-1}$, NO_3^--N (♦), NO_2^--N (Δ), SO_4^{2-}-S (○), S^{2-} (■) and N_2-N (**X**).

Despite the assay was performed under sulphide limiting conditions nitrate was fully reduced to nitrogen gas along the experiment which suggests that another sulphur source was used as electron donor (Table 3.6). Furthermore the amount of sulphate produced is similar to that predicted by stoichiometry in order to consume the initial amount of nitrate. Similar behaviour has been previously reported by Kleerebezem and Méndez (2002). These authors operated a fixed-film reactor under autotrophic denitrifying conditions with sulphide as electron donor. Their results show that the nitrogen recovery was almost complete while the sulphide recovery was not despite the sulphide was not detected in the effluent. They suggested the accumulation of another unknown sulphur product of the sulphide oxidation in the reactor. They confirmed this when they operated the reactor under sulphide limiting conditions and obtained an amount sulphate produced stoichiometrically according to the amount of nitrate consumed.

Table 3.6 Initial and final concentrations of substrates and products in the batch experiments performed with sulphide (S/NO_3^- of 0.15 mol·mol^{-1}) (Assay 2; Table 3.3)

NO_3^--N initial (mg·L^{-1})	S^{2-} initial (mg·L^{-1})	NO_3^--N final (mg·L^{-1})	S^{2-} final (mg·L^{-1})	N_2-N final (mg·L^{-1})	SO_4^{2-}-S final (mg·L^{-1})
45.0	16.0	0.0	0.0	47.6	138.3

The value of the specific ADA measured with sulphide was lower than that obtained with thiosulphate of 153 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}. This result suggests the occurrence of an inhibitory effect of sulphide on the denitrification activity, which agrees with results obtained by Beristain *et al*. (2006) and Beristain-Cardoso *et al.* (2009). They tested the denitrifying activity with different sulphur compounds and concluded that the denitrification rate strongly depended on the inorganic sulphur compound used as electron donor ($S_2O_3^{2-}$ >S^{2-}>>S^o). This behaviour confirms the fact that thiosulphate has an important role in the sulphur cycle, is easily biodegradable (Claus and Kutzner 1985), and it is the main sulphur source reported for enrichment of autotrophic denitrifying biomass (Fernández *et al*., 2006; Moon *et al*., 2004; Gu *et al*., 2004; Oh *et al*., 2004). On the other hand it is also known the toxic effect of sulphide over heterotrophic (Sorensen et al. 1980; and Knowels 1980) and autotrophic denitrifying micro-organisms (Goomers *et al*. 1988) which may explain the low achieved denitrification rates. The low autotrophic denitrifying rates found for elemental sulphur may be caused by its low solubility (Park and Yoo, 2009).

Results obtained from the experiment performed with an S/NO_3^- ratio of 0.7 $mol \cdot mol^{-1}$ (Assay 4; Table 3.3) indicated that nitrate was almost completely recovered as nitrogen gas while residual sulphide was detected at the end of the assay. Once more the amount of sulphate detected at the end of the experiment was higher than the initial added sulphide (Table 3.7) which agrees with results from the assay performed at a S/NO_3^- ratio of 0.15.

Table 3.7 Initial and final concentrations of substrates and products in the assay performed with sulphide (S/NO_3^- of 0.7 $mol \cdot mol^{-1}$) (Assay 4; Table 3.3)

NO_3^--N initial ($mg \cdot L^{-1}$)	S^{2-}-S initial ($mg \cdot L^{-1}$)	NO_3^--N final ($mg \cdot L^{-1}$)	S^{2-}-S final ($mg \cdot L^{-1}$)	N_2–N final ($mg \cdot L^{-1}$)	SO_4^{2-}-S final ($mg \cdot L^{-1}$)
28.0	45.0	1.75	7.0	21.4	60.0

It has been reported that during sulphide oxidation the main intermediate sulphur compound is the thiosulphate (Barbosa *et al*., 2006) as it was detected in the present experiment. The thiosulphate was transformed to sulphate before the end of the experiment (Figure 3.7).

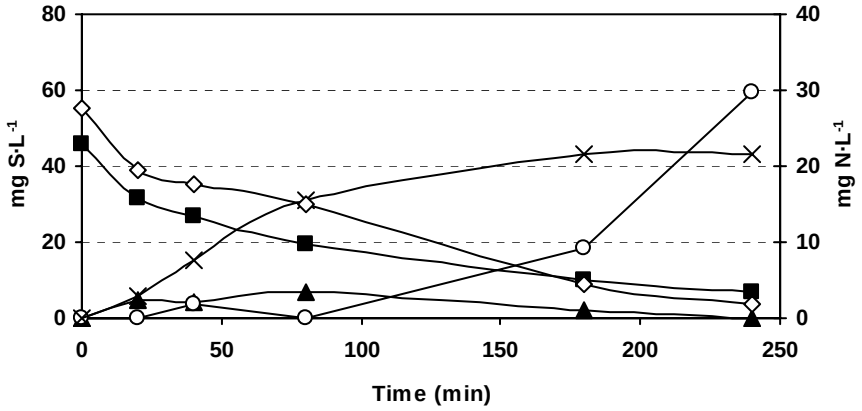

Figure 3.7 Evolution of nitrogen and sulphur compounds concentrations during the batch assay with sulphide at a S/N ratio of 1.6 $g \cdot g^{-1}$, S^{2-} (■), SO_4^{2-}-S (○), $S_2O_3^{2-}$-S (▲), NO_3^--N (◊) and N_2-N (**X**).

Measured specific ADA in this assay was of 106 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}. This value was lower than that found for the S/ NO_3^- ratio of 0.15. In previous works it has been observed that when the sulphide concentration increased the denitrifying activities decreased significantly. Beristain *et al.* (2006) studied the effect of the sulphide concentration on autotrophic denitrification. Their results showed that at low sulphide concentrations like 80 mg S^{2-} L^{-1} the denitrification occurred at a rate of 22.4 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}, while at 160 mg S^{2-} L^{-1} the denitrification rate decreased by nearly half of the previous on to 10.5 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} and almost by ten folds with 320 mg S^{2-} L^{-1} (1.4 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}). Beristain-Cardoso *et al.* (2009) found the same behaviour in the case of mixotrophic denitrifying biomass and they detected the transient formation of N_2O when high concentrations of sulphide were tested and suggested that the formation of this compound could explain the lost of denitrification. On the other hand, Gadekar *et al*. (2006) reported that the sulphide concentration also affected the sulphide oxidation and played an important role over the products of oxidation.

In the present experiment the SOA was of 169 mg S^{2-}(g VSS)$^{-1}$·d^{-1}, which is a value significantly lower than that measured in the experiments with thiosulphate of 643 mg $S_2O_3^{2-}$-S (g VSS)$^{-1}$·d^{-1}.

The assay to determine the specific ADA at the S/ NO_3^- ratio of 0.63 mol·mol^{-1} was performed following only gas production. The obtained value was of 131 mg NO_3^--N (g VSS)$^{-1}$ d^{-1}. This value was higher than that measured at the S/NO_3^- ratio of 0.7 mol·mol^{-1}.and smaller than that corresponding to the S/NO_3^- ratio of 0.15 mol·mol^{-1}.

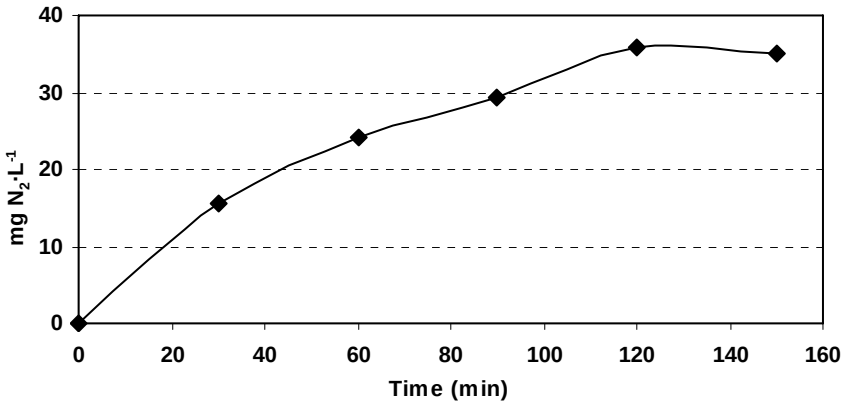

Figure 3.8 Nitrogen production at a S/NO_3^- ratio of 0.63 mol·mol^{-1}.

3.4.2.2.3 Specific ADA in the presence of elemental sulphur as electron donor (LPV)

In order to determine the specific ADA with elemental sulphur (Assay 5; Table 3.3) two assays were performed, 1) with 440 mg $S^o \cdot L^{-1}$ and 80 mg NO_3^--N·L^{-1}, and 2) a control without elemental sulphur following the nitrogen gas production. The nitrogen gas production profiles of both assays are shown in Figure 3.9. No significant difference in nitrogen production was detected between both assays. The nitrogen produced by the control experiment confirmed that another sulphur source has been used as electron donor for the nitrate reduction and suggested that the denitrification in the presence of elemental sulphur was not due to oxidation of this compound. This finding agrees with previously reported results by Kleerebezem and Méndez (2002). The values of the obtained specific ADA were of 12.5 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} for the control assay and of 11.5 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1} in the presence of elemental sulphur.

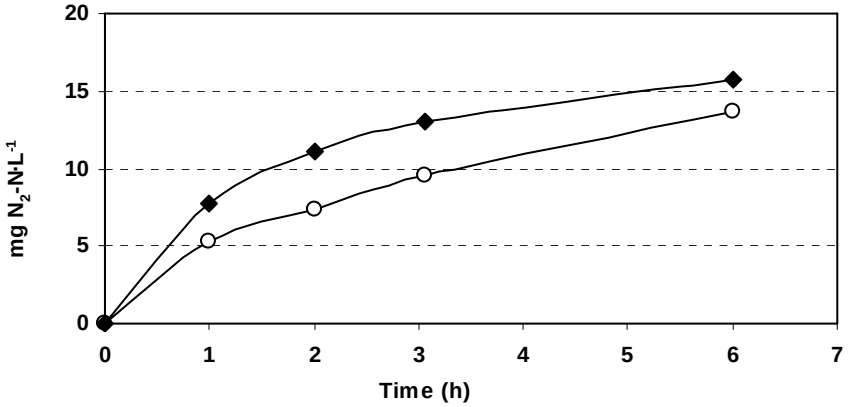

Figure 3.9 Profile of nitrogen production, control (○) and with S^o addition (♦).

3.4.2.2.4 Specific ADA in the presence of "stored" sulphur compound as electron donor (LPV)

According to the obtained results in the batch assays with sulphide as electron donor and the assay performed in absence of sulphur source (control for assay with elemental sulphur) the amount of sulphate produced was higher that the amount of applied sulphur compound. This observation suggested the presence of another sulphur compound formed during the oxidation of the sulphur specie tested. In order to clarify this aspect a batch

activity assay using the possible sulphur compound "stored" in the sludge (Assay 5, Table 3.3) was performed.

It has been reported that during the oxidation of reduced sulphur compounds many micro-organisms are able to form sulphur globules intra or extracellularly and later to use them as energy source for bicarbonate fixation (Janssen *et al.,* 1999; Nielsen *et al.,* 2000).

In order to demonstrate the presence of another sulphur compound stored in the sludge, two consecutive feedings of 52 mg NO_3^--N·L^{-1} were carried out in the batch test without external addition of the electron donor source. Results indicated that under these conditions the biomass was able to completely reduce the supplemented NO_3^--N·L^{-1} to nitrogen gas during the two consecutive feedings, despite no external sulphur compound source was supplemented (Figure 3.10). The denitrifying activities obtained for the two feedings were 119.5 and 123 mg NO_3^--N·(g VSS)$^{-1}$·d^{-1}, respectively.

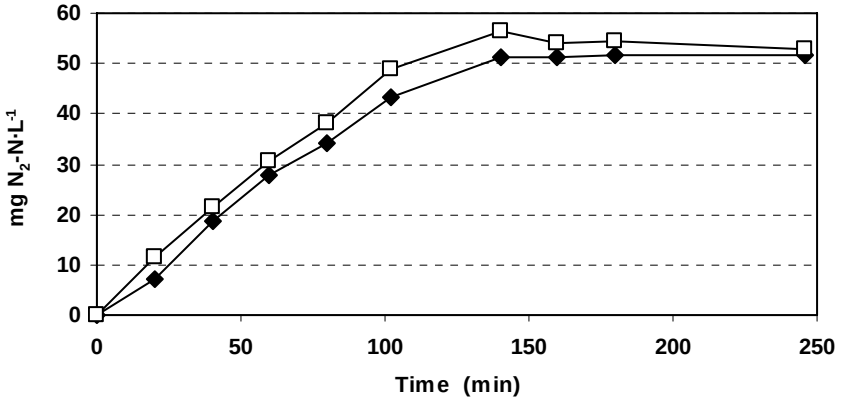

Figure 3.10 Profile of produced nitrogen gas during the two consecutive feedings with NO_3^--N·L^{-1} and without external sulphur source addition: first feeding (♦) and second feeding (□).

These results confirmed the presence of a certain sulphur compound which served as electron donor for reduction of nitrate to nitrogen gas. Similar results have been reported in the case of filamentous colourless bacteria *Thiotrix sp*. that in the presence of thiosulphate and in aerobic conditions were able to form sulphur globules inside the biomass which later disappeared when the biomass was exposed to anaerobic conditions

in the presence of nitrate as electron acceptor (Nielsen *et al*., 2000). Prange *et al.* (2002) characterized sulphur globules of different bacteria by X-ray absorption near edge structure spectroscopy (XANES) and they found that the composition of sulphur globules is closely related to the metabolism of different sulphur-oxidizing bacteria: sulphur rings (cyclooctasulphur) in the case of microaerobic chemotrophic sulphur-oxidizing bacteria, polythionates in the aerobic chemotrophic bacteria and sulphur chain in the anaerobically grown phototrophic sulphur bacteria.

The values of the denitrifying activities with the different sulphur compounds tested are resumed in Table 3.8. The values of the obtained denitrifying activities show the clear effect exerted by the used sulphur source. The maximum value was obtained with thiosulphate followed by sulphide and by elemental sulphur. These results agree with those previously reported by Beristain *et al*. (2006) who tested the autotrophic denitrification with thiosulphate, sulphide and elemental sulphur as electron donor and obtained values of nitrate degradation rates of 105.84, 23.1, and 11.2 mg NO_3^--N·d^{-1} respectively. The assay performed with the biomass using the "sulphur stored compound" presented an specific activity close to that obtained during the assay performed with sulphide at a S/N ratio of 1.45 g·g^{-1} and half than that measured when thiosulphate was used as electron donor.

Table 3.8 Specific ADA corresponding to the experiments performed with different sulphur compounds

Assay	Sulphur compound	S/NO_3^- ratio (mol·mol^{-1})	Specific ADA (mg NO_3^--N·(g VSS)$^{-1}$·d^{-1})
1	$S_2O_3^{2-}$	1.3	211
2	S^{2-}	0.15	153
3	S^{2-}	0.63	131
4	S^{2-}	0.7	106
5	S^o	2.4	11.5
	"S stored compound"		121

3.5. CONCLUSIONS

The results obtained in this work demonstrated that the strategy of batch feeding used to enrich autotrophic biomass favoured the development of the micro-organisms able to carry out the autotrophic denitrification at specific loads of 1.36 mg NO_3^--N (g VSS)$^{-1}$·d^{-1}.

Simultaneously the physical properties of the biomass were maintained in the adequate values (SVI value of 33.3 $mL \cdot (g\ VSS)^{-1}$) which allowed retaining a VSS concentration of 7.8 $g \cdot L^{-1}$ with a VSS/TSS ratio of 0.55 $g \cdot g^{-1}$.

The obtained enriched biomass was able to use different sulphur sources to carry out the autotrophic denitrification. The maximum specific autotrophic denitrifying activity (ADA) corresponded to the experiment with thiosulphate as electron donor and it was of 211 mg NO_3^--N·$(g\ VSS)^{-1} \cdot d^{-1}$. When sulphide was used the specific ADA was significantly reduced and influenced by S/NO_3^- ratio in such a way that the maximum obtained specific ADA was of 153 mg NO_3^--N·$(g\ VSS)^{-1} \cdot d^{-1}$ (S/NO_3^- of 0.15 $mol \cdot mol^{-1}$). The specific activity obtained with elemental sulphur was not conclusive due to the fact that the control without addition of external sulphur compound gave a similar value of specific ADA than the assay with sulphur presence of 12.5 mg NO_3^--N·$(g\ VSS)^{-1} \cdot d^{-1}$ and 11.5 mg NO_3^--N·$(g\ VSS)^{-1} \cdot d^{-1}$, respectively. The presence of a sulphur source stored in the sludge which served as electron donor for the nitrate reduction was confirmed by the obtained value of the specific ADA of 121 mg NO_3^--N·$(g\ VSS)^{-1} \cdot d^{-1}$ without sulphur source addition.

3.6. REFERENCES

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Chapter 4.

Autotrophic Denitrification with Sulphide in a Sequencing Batch Reactor

Summary

In this chapter a sequencing batch reactor was used to simultaneously remove both sulphide and nitrate via an autotrophic denitrification process. The sulphide loading rates were gradually increased from 200 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$ to 450 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$ while the nitrogen loading rates were kept constant at 450 mg NO_3^--$N\cdot L^{-1}\cdot d^{-1}$.

The obtained results demonstrated that it was possible to carry out autotrophic denitrification in a sequencing batch reactor with removal efficiencies of sulphide and nitrogen of 100% and 67%, respectively. The efficiency of the process was influenced by the pH value in the reactor. The operation at pH values higher than 9.0 decreased the efficiency of sulphide oxidation into sulphate to 5.2%. The initial specific biomass activity was of 170 mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$ and decreased down to 15.4 mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$ when the system was operated with pH values higher than 9. During the last operational stages, the pH was controlled at a value around 8 which allowed the restoration of the specific biomass activity to cope with the fed sulphide loads.

The results obtained in this chapter have been included in:
Fajardo C. *et al*. (XXX). Autotrophic Denitrification with sulphide in a Sequencing Batch Reactor. Journal of Environmental Management, (in press).

4.1 INTRODUCTION

The most common pollutants present in the water are compounds containing organic carbon, nitrogen, sulphur and phosphorus. These compounds are frequently removed from the wastewater by means of combined biological processes like nitrification, denitrification and/or sulphate reduction (Henze *et al.*, 2002).

Autotrophic denitrification can be used to remove both nitrogen oxides and sulphur compounds from wastewater, being an interesting alternative to heterotrophic denitrification for wastewaters with high nitrate concentration and low organic matter content (Kuai and Verstraete, 1999; Vaiopoulou *et al*., 2005). This process is carried out by autotrophic sulphur bacteria like *Thiobacillus denitrificans* which are able to use reduced sulphur compounds (S^{2-}, S^{o}, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}) as electron donors and nitrate as electron acceptor under anoxic conditions (Robertson and Kuenen, 1992). When industrial wastewaters containing both ammonia and sulphate are treated by means of anaerobic digestion, the generated biogas is rich in hydrogen sulphide that needs to be removed to enable further uses of the biogas (Kantachote *et al.,* 2008). Nitrogen removal is generally achieved by subsequent nitrification and denitrification of the effluent from the anaerobic reactor (Chernicharo and Nascimento, 2001; Foresti *et al.,* 2006). Since the effluent from the anaerobic digester has not enough organic matter to carry out heterotrophic denitrification, the hydrogen sulphide present in the biogas can be used as electron donor in an autotrophic post-denitrification step system (Kleerebezem and Méndez, 2002). In this process nitrate is reduced into nitrogen gas while sulphide is oxidized into sulphate or sulphur according to Equations [4.1] and [4.2] (Moon *et al*., 2004; Manconi *et al*., 2006; Lau *et al*., 2006).

$$S^{2-} + 1.6\ NO_3^- + 1.6\ H^+ \rightarrow SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O \quad \Delta G° = -743.9\ kJ\ mol^{-1} \qquad [4.1]$$

$$S^{2-} + 0.4\ NO_3^- + 2.4\ H^+ \rightarrow S^o + 0.2\ N_2 + 1.2\ H_2O \quad \Delta G° = -191.0\ kJ\ mol^{-1} \qquad [4.2]$$

An important number of research works on autotrophic denitrification have been focused on the determination of kinetic parameters in axenic and mixed cultures at different S/N ratios and in the presence of sulphide, nitrite, nitrate and COD (Gadekar *et*

al., 2006; Wang *et al*., 2005; Oh *et al.,* 2000; Campos *et al.*, 2008). It has been reported that both substrates and products of the autotrophic denitrification may cause inhibitory effects. Inhibition by sulphate has been reported at concentrations of 1.5 g SO_4^{2-}-S·L^{-1}, while nitrite appears to be a strong inhibitor of denitrification even at low concentrations (100 mg NO_2^--N·L^{-1}) (Claus and Kutzner, 1985; Krishnakumar and Manilal 1999; Oh *et al*., 2000; Campos *et al*., 2008).

Autotrophic denitrification using different sulphur compounds has been carried out with different kinds of technologies (Table 4.1). The application of packed bed reactors to this process has been limited by clogging problems due to the excessive biomass growth, the formation of anaerobic microenvironments and the accumulation of nitrite as a by-product (Kim *et al*., 2004; Gu *et al*., 2004; Moon *et al*., 2004). The use of continuous stirred tank reactors (CSTRs) with suspended biomass could be a good alternative to avoid these problems. However this kind of systems requires a long start-up period due their poor biomass retention capacity (Gadekar *et al*., 2006; Wang *et al*., 2005; Kim *et al*., 2004).

The Sequencing Batch Reactor (SBR) is a suitable system to enrich bacterial populations with low growth rates thanks to the strong selective conditions achieved in this system and the high retention of growing biomass (90%) (Strous *et al.*, 1998). Another advantage of the SBRs is the feasibility to change the operational strategy to optimise the efficiency (Humphreys and Banks, 1995) and the easy control of the process (Andreottola *et al.*, 2001). Nevertheless, limited information is available on the use of this technology applied to autotrophic denitrification (Pérez *et al*., 2007).

Table 4.1 Autotrophic Denitrifying Reactors.

Reactor	Electron donor	SLR (mg $S \cdot L^{-1} \cdot d^{-1}$)	NLR (mg $N \cdot L^{-1} \cdot d^{-1}$)	S/N (mol/mol)	Products	Removal efficiency (%)	Reference
Packed bed reactor	S°		240		SO_4^{2-}, NO_2^-	95% NO_3^-	Soares, 2002
Packed bed reactor	S°		190		SO_4^{2-}, NO_2^-	97% NO_3^-	Gu *et al.*, 2004
Packed bed reactor	S°		120		SO_4^{2-}	100% NO_3^-	Moon *et al*., 2004
Membrane reactor	S°		310		SO_4^{2-}, NO_2^-	80% NO_3^-	Kimura *et al.*, 2002
Fluidized bed reactor	S°		2530		SO_4^{2-}	90% NO_3^-	Kim *et al.*, 2004
Packed bed reactor	S°		240		SO_4^{2-}	96% NO_3^-	Sierra-Alvarez *et al*., 2007
CSTR	S^{2-}	300		2.3	SO_4^2, Sº	100% S^{2-}, NO_3^-	Manconi *et al*., 2007
Packed bed reactor	S^{2-}	3000		<0.62	SO_4^{2-}, Sº	100% S^{2-}, NO_3^-	Kleerebezem and Méndez, 2002
CSTR	S^{2-}	1220	640	2.0	NO_2^-, Sº	100% S^{2-}	Gadekar *et al*., 2006
Fluidized bed reactor	S^{2-}	600		2.5	S°	100% S^{2-}, 95% NO_3^-	Wang *et al.*, 2005
CSTR	$S_2O_3^{2-}$	980	400	1.0	SO_4^{2-}	95% $S_2O_3^{2-}$, 100% NO_3^-	Manconi *et al*., 2007
Packed bed reactor	$S_2O_3^{2-}$		6300	3.3	SO_4^{2-}	90% NO_3^-	Yamamoto-Ikemoto *et al*., 2000

NLR: Nitrogen loading rate

SLR: Sulphide loading rate

4.2 OBJECTIVE

The objectives of this work were to test the operation of an autotrophic denitrifying sequencing batch reactor fed with different sulphide loading rates and to characterize the properties of the obtained biomass.

4.3 MATERIALS AND METHODS

4.3.1 Experimental set-up

The process was carried out in a SBR with a working volume of 1 L. The reactor was inoculated with 750 mL of sludge (5.75 g $VSS \cdot L^{-1}$) previously operated in a batch reactor fed with thiosulphate and nitrate (Fajardo *et al*., 2008). The temperature of operation was maintained at 30 ± 1ºC by means of a thermostatic jacket. Complete mixture inside the reactor was achieved with a mechanical stirrer operated at 150 rpm. The hydraulic retention time (HRT) was fixed in 1 day. The reactor was flushed with a mixture of 95% Ar and 5% CO_2 to maintain anoxic conditions (Figure 4.1).

Figure 4.1 Autotrophic denitrifying SBR.

The SBR was operated in cycles of 6 h controlled by means of a PLC (CPU224, Siemens). The operational cycle comprised four phases: feeding in stirring conditions (300 min), stirring (30 min), settling (15 min) and effluent withdrawal (15 min). A volumetric exchange ratio of 25% was applied.

4.3.2 Feeding media

The reactor was fed with a synthetic medium composed by two solutions. Solution A consisted of ($g \cdot L^{-1}$): $NaHCO_3$ (3.0) and sulphide supplied as $Na_2S \cdot 3H_2O$ (1.65 – 3.71 $g \cdot L^{-1}$) and adjusted with HCl 1 M to pH 8. Solution B consisted of ($g \cdot L^{-1}$): $NaNO_3$ (5.46), Na_2HPO_4 (3.0), KH_2PO_4 (0.6 and 3.6), NH_4Cl (0.2), $MgSO_4$ anhydrous (0.08) and 2 mL per litre of a traces solution. The traces solution contained ($g \cdot L^{-1}$): $Na_2MoO_4 \cdot 2H_2O$ (1.0), $FeSO_4 \cdot 7H_2O$ (38.0), $CaCO_3$ (2.0), $ZnSO_4 \cdot 7H_2O$ (1.5), $MnCl_2 \cdot 4H_2O$ (1.0), $CuSO_4 \cdot 5H_2O$ (0.25), $CoCl_2 \cdot 6H_2O$ (0,25), $NiCl_2 \cdot 6H_2O$ (0.25), H_3BO_3 (0.5) and HCl (156.2). Solutions A and B were prepared separately and mixed in equal amounts to prepare the feeding medium.

The reactor was operated under sulphide limiting conditions (Table 4.2) since the molar S/N ratio was always lower than the stoichiometric ratio of 0.625 S^{2-}/NO_3^- (Equation [4.1]). The sulphide loading rate applied to the system was changed by varying the sulphide inlet concentration while nitrate concentration was maintained constant at a value of 450 mg NO_3^--$N \cdot L^{-1}$.

Table 4.2 Operational conditions applied to the SBR.

Stage	Days	S^{2-}/NO_3^- (mol/mol)	SLR (mg $S^{2-} \cdot L^{-1} \cdot d^{-1}$)
I	0-27	0.20	200
II	28-48	0.25	250
III	49-62	0.30	300
IV	63-76	0.20	200
V	77-98	0.10	100
VI	99-128	0.15	150
VII	129-166	0.30	300
VIII	167-195	0.35	350
IX	196-220	0.45	450

4.3.3 Analytical methods

Analytical methods applied to monitor the reactor performance as well as biomass activities are described in Chapter 2.

4.3.4 Calculations

4.3.4.1 Mass balance of sulphur compounds

The possible formation or consumption of elemental sulphur (intermediate product) during the process can be estimated as the difference between the amount of sulphur compounds in the influent and that of the effluent [4.3]:

$$S^0\ (mgS \cdot d^{-1}) = Q \cdot \left(\left(\left[S^{2-} - S \right]_i + \left[SO_4^{2-} - S \right]_i \right) - \left(\left[S^{2-} - S \right]_e + \left[SO_4^{2-} - S \right]_e \right) \right) \qquad [4.3]$$

Where:

Q: flow rate ($L \cdot d^{-1}$)

$[S^{2-}S]_i$, $[S^{2-}S]_e$: sulphide concentration in the influent and effluent (mg $S \cdot L^{-1}$)

$[SO_4^{2-}S]_i$, $[SO_4^{2-}S]_e$: sulphate concentration in the influent and effluent (mg $S \cdot L^{-1}$)

The percentage of sulphate generated from sulphide consumption was calculated according to equation [4.4]:

$$\%SO_4^{2-} - S\ produced = \frac{\left[SO_4^{2-} - S\right]_e - \left[SO_4^{2-} - S\right]_i}{\left[S^{2-} - S\right]_i - \left[S^{2-} - S\right]_e} \cdot 100 \qquad [4.4]$$

4.3.4.2 Nitrite acumulation

Nitrite is an intermediate product of nitrate reduction and its production percentage was estimated according to equation [4.5]:

$$\%NO_2^- - N\ produced = \frac{\left[NO_2^- - N\right]_e}{\left[NO_3^- - N\right]_i - \left[NO_3^- - N\right]_e} \cdot 100 \qquad [4.5]$$

Where:

$[NO_3^-N]_i$, $[NO_3^-N]_e$: nitrate concentration in the influent and effluent (mg $N \cdot L^{-1}$)

$[NO_2^-N]_e$: nitrite concentration in the effluent (mg $N \cdot L^{-1}$)

4.3.4.3 Electron balances

The electron balances were calculated for each operational stage taking into account the possible electron donor and electron acceptor reactions and assuming the anabolic

reactions as negligible due to the low yield coefficient of the autotrophic denitrifying bacteria (Table 4.4).

Table 4.4 Possible electron donor and electron acceptor reactions during autotrophic denitrification with sulphide and nitrate.

Electron donor reactions
$HS^- \rightarrow S^0 + H^+ + 2e^-$
$HS^- + 4H_2O \rightarrow SO_4^{2-} + 9H^+ + 8e^-$
Electron acceptor reactions
$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$
$NO_3^- + 6H^+ + 5e^- \rightarrow 0.5N_2 + 3H_2O$

In order to calculate the number of electrons donated, the possible formation or consumption of elemental sulphur was considered. When Sº was generated the number of electrons donated was calculated by equation [4.6] while when Sº consumption was detected this number was calculated by equation [4.7]:

$$e^- \text{ donated } (e^- \text{ mol}\cdot d^{-1}) = 2\cdot\frac{Q\cdot(([S^{-2}-S]_i+[SO_4^{2-}-S]_i)-([S^{-2}-S]_e+[SO_4^{2-}-S]_e))}{32}+8\cdot\frac{Q\cdot([SO_4^{2-}-S]_e-[SO_4^{2-}-S]_i)}{32} \quad [4.6]$$

$$e^- \text{ donated } (e^- \text{ mol}\cdot d^{-1}) = 2\cdot\frac{Q\cdot(([S^{-2}-S]_i+[SO_4^{2-}-S]_i)-([S^{-2}-S]_e+[SO_4^{2-}-S]_e))}{32}+8\cdot\frac{Q\cdot([S_4^{2-}-S]_i-[S^{2-}-S]_e)}{32} \quad [4.7]$$

Electrons accepted were estimated according to equation [4.8]:

$$e^- \text{ accepted } (e^- \text{ mol} \cdot d^{-1}) = 5 \cdot \frac{Q \cdot \left(\left[NO_3^- - N \right]_i - \left[NO_3^- - N \right]_e - \left[NO_2^- - N \right]_e \right)}{14} + 2 \cdot \frac{Q \cdot \left[NO_2^- - N \right]_e}{14} \quad [4.8]$$

4.3.4.4 Biomass balance

The biomass yield coefficient was estimated for each operational stage taken into account the amount of biomass generated referred to the amount of nitrogen consumed (equation [4.9]):

$$Y = \frac{\Delta X_r \cdot V_r + \overline{X}_e \cdot Q \cdot \Delta t}{Q \cdot \left(\left[NO_3^- - N \right]_i - \left[NO_3^- - N \right]_e - \left[NO_2^- - N \right]_e \right)} \quad [4.9]$$

Where:

Y: biomass yield coefficient (g VSS·g N^{-1})

ΔX_r: Change of biomass concentration inside the reactor during the operational stage (g VSS·L^{-1})

V_r: Volume of the reactor (L)

X_e: Average value of biomass concentration in the effluent during the operational stage (g VSS·L^{-1})

Δt: Duration of the considered operational stage (d)

4.4 RESULTS AND DISCUSSION

4.4.1 Reactor operation

4.4.1.1 Sulphide removal

The reactor was operated during 220 days in nine steps under the conditions previously described in Table 4.2. During Stage I the reactor was firstly fed with a synthetic medium containing 200 mg $S^{2-} \cdot L^{-1}$ and 450 mg NO_3^--N·L^{-1}. In this stage the amount of sulphate produced was greater than that expected from the stoichiometry (Equation [4.1]) (Figure 4.2). This behaviour was already observed in the batch assays carried out with the biomass used as inoculum of the reactor. This phenomenon could be attributed to a possible release of previously accumulated elemental sulphur inside cells (Kleerebezem and Méndez, 2000; Gommers *et al*., 1988).

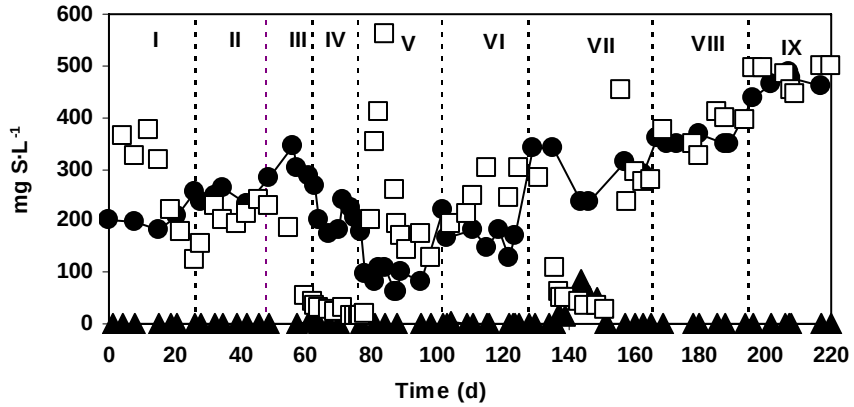

Figure 4.2 Evolution of the concentrations of S^{2-} in the influent (●) and S^{2-} (▲) and SO_4^{2-}-S (□) in the effluent.

Taking into account that all the fed sulphide was oxidized into sulphate, during Stage II its concentration was increased up to 250 mg $S^{2-} \cdot L^{-1}$. At this point the amount of sulphate produced was correlated with the stoichiometric expected value (Figure 4.2).

A new increase of sulphide concentration up to 300 mg $S^{2-}\cdot L^{-1}$ was applied in Stage III. Under this condition no sulphide was detected in the effluent, but a decrease in the sulphate production was observed together with values of pH around 8.6 (Table 4.3). These results agree with those obtained by Mahmood *et al.* (2008) who observed that pH values higher than 9 did not affect the sulphide removal efficiency but led to a decreased of sulphate production. Such results indicate that other intermediate sulphur compounds (elemental sulphur, thiosulphate or polysulphide) could be generated when the reactor is operated under alkaline conditions.

During Stage IV the concentration of sulphide in the feeding was reduced (200 mg $S^{2-}\cdot L^{-1}$) to recover the efficiency of the reactor. At this moment an average value of pH of 9.3 was registered. In the effluent neither sulphide nor sulphate were detected. The liquid media inside the reactor presented a milky appearance (Figure 4.3A). The microscopic observation of the sludge showed the presence of white precipitates on its surface (Figure 4.3B). Elemental composition analysis showed these precipitates were mainly composed by elemental sulphur. Elemental analysis of sludge gave as result a sulphur content between 0.071-0.113 g $S^{o}\cdot(g\ VSS)^{-1}$. These values were higher than those reported by Gommers *et al*. (1988). Several authors also found the formation of these elemental sulphur precipitates which would be oxidized to produce sulphate if sufficient nitrate was present in the system (Manconi *et al*., 2007; Beristain *et al.,* 2006; Kleerebezem and Méndez, 2002). Furthermore sulphur globules inside the biomass are formed to be used as energy source for bicarbonate fixation (Janssen *et al.,* 1999; Nielsen *et al.,* 2000). Associated to this fact a significant biomass wash-out occurred during this period and the solids concentration in the effluent increased from 15 to 60 mg of $VSS\cdot L^{-1}$.

In stage V the inlet sulphide concentration was decreased again down to 100 mg $S^{2-}\cdot L^{-1}$ and the pH value of the solution B was fixed with HCl at 6 which allowed maintaining a pH value inside the reactor around 7.5. Under these conditions the system re-established its previous state of fully sulphide to sulphate conversion. At this moment a non stoichiometric amount of sulphate up to 550 mg $SO_4^{2-}\text{-}S\cdot L^{-1}$ was produced compared to the amount of sulphide in the feeding (100 mg $S^{2-}\cdot L^{-1}$). The excess of sulphate formed can be a product of the oxidation of the accumulated elemental sulphur in the previous stage. During this stage the concentration of sulphate in the effluent gradually decreased to reach the concentration expected from the stoichiometry.

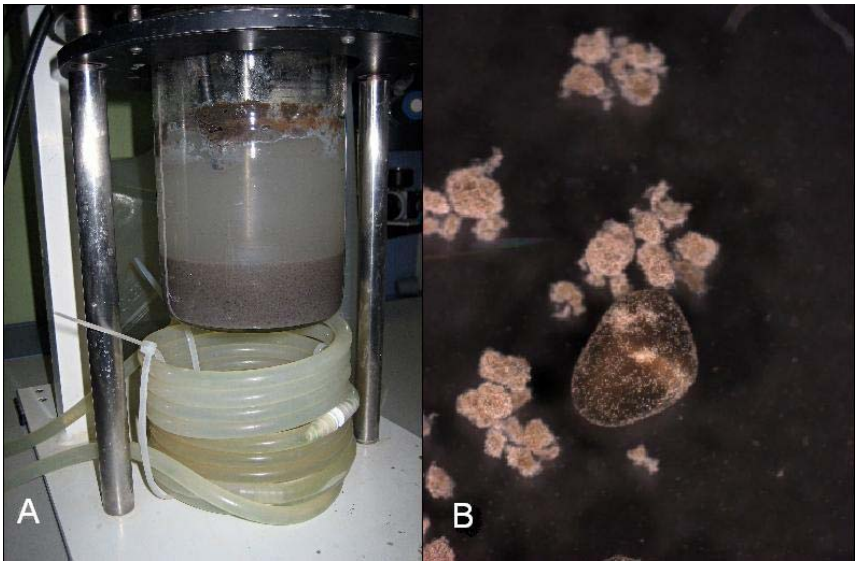

Figure 4.3 Images of the reactor and the sludge during Stage IV (pH of the effluent of 9.3). Image A) milky appearance of liquid media and; image B) micrograph of sludge with white precipitates on its surface (25x).

In stage VI the sulphide concentration was increased to 150 mg $S^{2-}{\cdot}L^{-1}$ while the complete oxidation of sulphide into sulphate was maintained. In Stage VII the inlet sulphide concentration was increased up to 300 mg $S^{2-}{\cdot}L^{-1}$ which caused a sudden increase of the pH value inside the reactor up to 10 (data non shown). Under these conditions, sulphate production was almost depleted and the system was not able to remove all the applied sulphide. In order to control the pH value around 7.5 inside the reactor, the composition of solution A was changed by increasing the concentration of KH_2PO_4 up to 3.6 $g{\cdot}L^{-1}$. This strategy allowed restoring both sulphide removal efficiency and sulphate production.

During the next stages it was possible to increase the feeding sulphide concentrations up to 450 mg $S^{2-}{\cdot}L^{-1}$ (Stage IX) with full oxidation of sulphide into sulphate.

4.4.1.2 Sulphur compounds mass balance

Mass balances of sulphur compounds were calculated to detect the possible formation or consumption of elemental sulphur during the operational period (Figure 4.4). No formation of elemental sulphur was detected in those stages when the average pH value was kept between 7.5-8.0 (Stages I, V, VI, VIII and IX) (Figure 4.4). However when pH registered values close to 9 (Stages II, III, IV and VII) the system was not able to fully oxidize sulphide into sulphate and the efficiency of sulphate formation decreased down to a minimum value of 5.2%. This behaviour has been widely reported in autotrophic denitrifying systems fed with medium containing sulphide (Manconi *et al*., 2006; Kleerebezem and Méndez, 2002; Beristain *et al.,* 2006). Mahmood *et al.* (2008) observed that at loading rates of 1.15 g $S^{2-}{\cdot}L^{-1}{\cdot}d^{-1}$ and working at pH 9.85, registered sulphide removal efficiency was of 99.7% but only 6.3% of this sulphide was recovered as sulphate.

During the first days of Stage V the pH declined abruptly to values lower than 6 due to the addition of HCl to the feeding media. These low pH values caused a sudden increase of sulphate production which exceeded that expected from the sulphide supplied to the system. This fact can be attributed to the oxidation of sulphur accumulated during Stages III and IV. Sulphate production also exceeded sulphide consumption during Stage I, probably due to the presence of elemental sulphur in the biomass used as inoculum.

At end of the operational period a SLR of 450 mg $S^{2-}{\cdot}L^{-1}{\cdot}d^{-1}$ was fully oxidized into sulphate at efficiencies close to 100%. Similar efficiencies have been found by Vaiopoulou *et al.* (2005) working with SLR of 165 mg $S^{2-}{\cdot}L^{-1}{\cdot}d^{-1}$. However Manconi *et al.* (2007) observed that only of 50% of the sulphide was recovered as sulphate at a SLR of 280 mg $S^{2-}{\cdot}L^{-1}{\cdot}d^{-1}$. Previous studies showed that autotrophic denitrification using sulphide as electron donor is generally limited by the rate of elemental sulphur oxidation and that high conversions of sulphide into sulphate were not achieved at SLR higher than 300 mg $S^{2-}{\cdot}L^{-1}{\cdot}d^{-1}$ (Kleerebezem and Méndez, 2002; Wang *et al.,* 2005; Mahmood *et al.,* 2007, 2008).

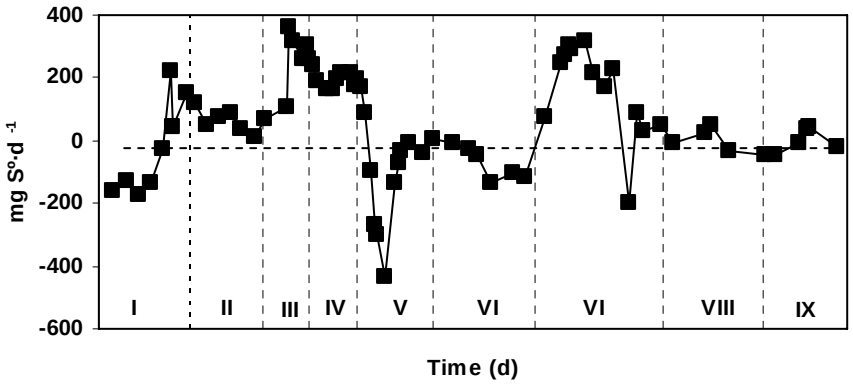

Figure 4.4 Estimated generation and/or consumption of elemental sulphur.

Table 4.3 Performance of the SBR at different sulphide loading rates. The number of samples used to calculate the media values is indicated between brackets.

Stage	Days	pH effluent	S^{2-} Removed (%)	SO_4^{2-}-S Produced $(S^{2-}_{Removed})^{-1}$ (%)	NO_3^--N Removed (%)	NO_2^--N Produced $(NO_3^-\text{-N}_{Removed})^{-1}$ (%)
I	0 - 27	7.8 ± 0.4(14)	100 ± 0.1(8)	75.8 ± 37.5(3)	15.6 ± 15.0(7)	37.1 ± 34.0(4)
II	28 - 48	8.0 ±0.3(14)	100 ± 0.1(6)	74.3 ± 16.0(6)	36.4 ± 10.7(6)	68.0 ± 24.7(6)
III	49 - 62	8.6 ± 1.4(7)	99.0 ± 0.6(3)	37.0 ± 32.2(4)	21.8 ± 12.1(5)	26.8 ± 14.6(2)
IV	63 - 76	9.3 ± 0.2(9)	97.0 ± 4.0(5)	5.2 ± 3.6(8)	19.7 ± 5.2(8)	27.5 ± 22.8(8)
V	77 - 98	7.5 ±1.3(10)	100 ± 0.1(5)	208.2 ± 16.4(12)	30.1 ± 19.5(11)	3.1 ± 2.5(7)
VI	99 - 128	8.2 ±0.7(13)	99.0 ± 0.4(9)	136.4 ± 24.0(6)	37.1 ± 5.6(7)	52.3 ± 27.0(7)
VII	129 - 166	8.4 ± 0.9(24)	94.0 ± 12.4(11)	51.7 ± 51.5(14)	40.3 ± 12.0(12)	3.0 ± 2.0(10)
VIII	167 - 195	7.7 ± 0.4(14)	100 ± 0.1(6)	102.2 ± 8.0(6)	46.8 ± 5.0(6)	33.4 ± 14.4(6)
IX	196 - 220	7.7 ± 0.2(18)	100 ± 0.1(5)	100.0 ± 9.0(8)	66.9 ± 9.7(9)	27.5 ± 16.2(7)

4.4.2 Nitrogen removal

Since the operation of the reactor was carried out under limiting sulphide conditions the complete nitrate removal was not achieved during the whole operational period (Figure 4.5). This fact caused that the nitrate removal efficiency increased as the S/N ratio of the feeding medium was increased (Figure 4.6). On the other hand, nitrite formation was observed during most of the operational stages in different amounts. This nitrite accumulation could be explained by two factors: 1) the higher value of the specific utilization rate of nitrate compared to that of nitrite which controls the overall consumption rate of nitrogenous compounds (Blécon *et al.*, 1983; Campos *et al.*, 2008) and; 2) the limitation of sulphide which hinders the latter consumption of the accumulated nitrite (Campos *et al.*, 2008). Therefore, an increase of nitrate removed would cause an increase in the nitrite generated as it can be observed in Figure 4.7.

Nitrite can exert an inhibitory effect on the autotrophic denitrification process. In fact, detrimental effects of this compound on the sulphide oxidation rate were detected by Krishnakumar and Manilal (1999) and Claus and Kutzner (1985) at concentrations of 30 and 60 mg NO_2^--N·L^{-1}, respectively. However, Campos *et al.* (2008) only found inhibitory effects at concentrations higher than 60 mg NO_2^--N·L^{-1} when thiosulphate was used as electron donor. In the present work, although the concentration of nitrite reached values higher than 100 mg NO_2^--N·L^{-1} during several operational stages, no effects of this compound neither on the sulphide oxidation efficiency nor on the biomass activity (see section 4.4.5) were observed.

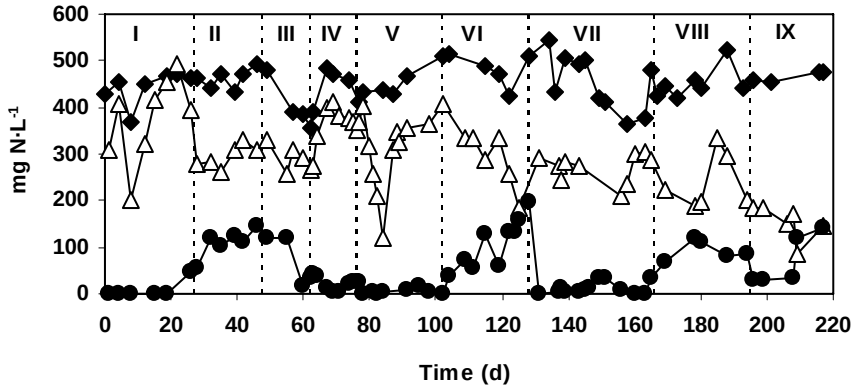

Figure 4.5 Behaviour of nitrogen compounds NO_3^--N influent (♦), NO_3^--N effluent (Δ) and NO_2^--N effluent (●).

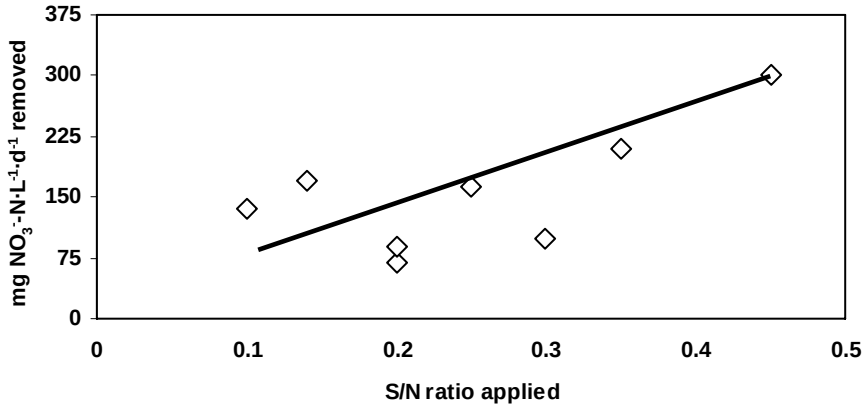

Figure 4.6 Nitrate loading removed at different S/N ratios.

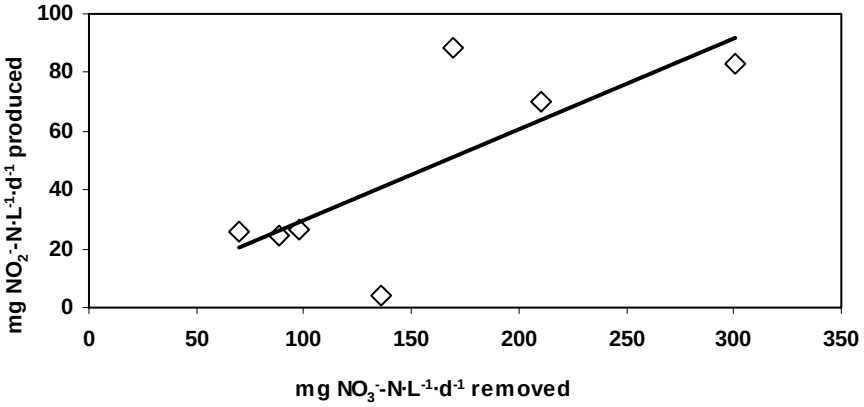

Figure 4.7 Nitrite production in relation to the nitrate removed.

4.4.3 Electron balances

The electron balances over the reactor were calculated considering the number of electrons potentially accepted or donated per day (Equations [4.6], [4.7] and [4.8]). To estimate the electrons donated it was assumed that all the sulphide removed, but not necessarily recovered as sulphate, was converted to elemental sulphur during Stages III, IV and VII. When sulphate production exceeded sulphide consumption (Stage V) it was considered that the excess of sulphate was generated from the previously accumulated elemental sulphur (Table 4.6). In spite of these assumptions, there are differences between the electrons donated and the electrons accepted. The obtained values are lower than 30% which indicates that in most cases the electron balances fit appropriately.

Table 4.4 Electron balances

S/N mol/mol	e^- donated ($e^- \cdot mol \cdot d^{-1}$)	e^- accepted ($e^- \cdot mol \cdot d^{-1}$)	$\triangle e^-$ (e^- donated)$^{-1}$ (%)
0.20	46.5 ± 6.8	36.6 ± 21.0	21.3 ± 47.0
0.25	53.7 ± 5.7	44.5 ± 13.2	17.1 ± 26.5
0.30	42.0 ± 17.3	35.4 ± 12.2	15.7 ± 50.7
0.10	47.1 ± 30.0	50.8 ± 34.7	-7.8 ± 97.5
0.14	57.0 ± 8.4	52.8 ± 9.6	7.3 ± 22.4
0.35	92.5 ± 6.3	66.7 ± 15.0	28.0 ± 17.7
0.45	117.7 ± 5.0	97.7 ± 7.8	16.5 ± 8.0

4.4.4 Evolution of the biomass concentration and its physical properties

The concentration of solids in the effluent was practically constant during stages with stable sulphide oxidation to sulphate and its value was around 20 mg VSS·L^{-1}. At end of Stage III a suddenly increment of pH close to 10 was registered which provoked the biomass wash-out and the solids concentrations in the effluent increased up to 60 mg·L^{-1}. Once the pH value was restored to values around 7.5 the solids concentration decreased to 20 mg VSS·L^{-1}. During Stage VI this phenomenon increased in importance but it did not affect the biomass concentration inside the reactor (Figure 4.8).

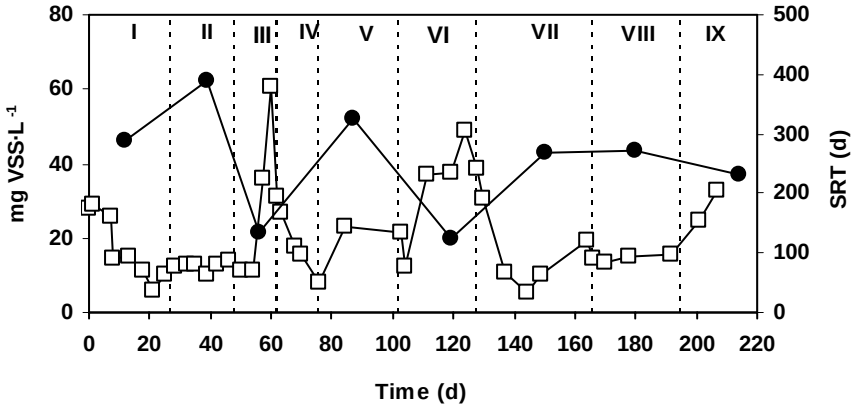

Figure 4.8 Evolution of VSS concentration in the effluent (□) and solids retention time (SRT) (●).

During the whole operational period the biomass concentration in the reactor was almost constant with an average value of 4.54 g VSS·L^{-1} (Figure 4.9) and presented a VSS/TSS ratio of 0.72 indicating a certain content of inorganics inside the reactor. This value is similar to that of 0.6-0.7 reported by Chen *et al*. (2008) for enriched autotrophic denitrifying sludge fed with sulphide. Solids retention time (SRT) was most part of the operational period higher than 200 d and its value only decreased until 133 d and 124 d during Stages III and VI, respectively, due to the wash-out episodes (Figure 4.8). The estimated SRT values during the whole operational periods were high enough to guarantee that no relevant loss of biomass occurred during the wash-out events and that the biomass concentration inside the reactor remained almost constant.

The sludge volumetric index (SVI) of the biomass changed slightly with respect to the inoculum but it was maintained always in appropriated values. Its average value was of 77.6 mL·(g VSS)$^{-1}$ (Figure 4.9). The biomass yield values obtained, according to equation (9), were between 0.11 and 0.21 mg VSS·(mg NO_3^--N)$^{-1}$ which are similar to those previously reported by Kim and Son (2000) and Manconi *et al*. (2007) for this kind of biomass.

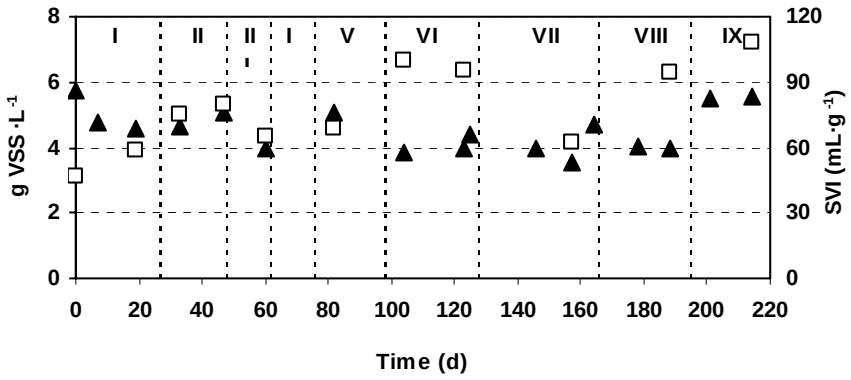

Figure 4.9 Concentration of VSS in the reactor (▲) and SVI (□).

4.4.5 Activity measurements

During each operational stage the maximum specific activity of the biomass was determined by means of batch experiments (Figure 4.10). Until day 62 of operation when concentrations of sulphide in the influent were between 200 and 300 mg $S^{2-}\cdot L^{-1}$, the maximum specific sulphide oxidizing activities remained constant around an average value of 100 mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$.

During Stages IV and VII, the maximum specific activities decreased down to 15.4 mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$ and 24.6 mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$, respectively, problably due to the high pH values inside the reactor. These specific activities were lower than the specific applied SLR which would explain why not full sulphide removal efficiencies were observed during both stages (Figure 4.10). Krishnakumar and Manilal (1999) reported that the operation at pH values of 8.5 and 9.5 caused the reduction of the sulphide oxidizing efficiencies down to 50% and 20%, respectively. When the pH value was controlled the full efficiency of the system was gradually recovered and the maximum specific activity increased up to 318 mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$ (Stage IX). This value is close to that found by Beristain-Cardoso *et al.* (2009) in batch assays.

The specific applied SLR to the reactor during most part of operational period was lower than the measured maximum specific activities of the biomass. This fact was in accordance to the high removal efficiencies observed for this compound (Figure 4.10).

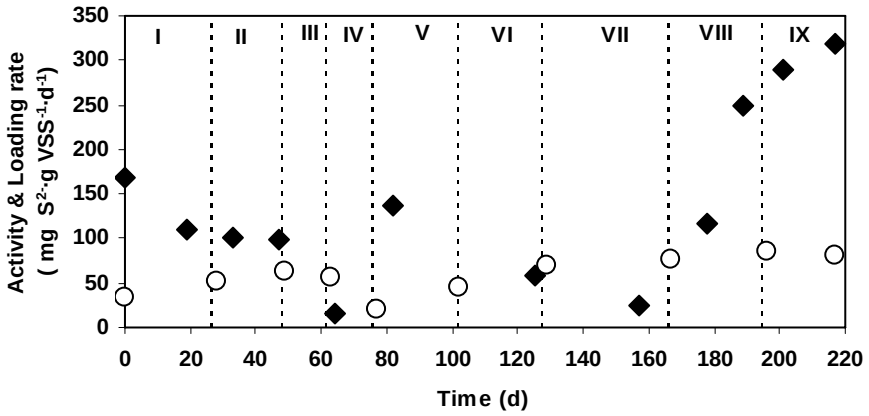

Figure 4.10 Maximum specific sulphide oxidizing activity (♦) and specific SLR (○) in mg $S^{2-}\cdot(g\ VSS)^{-1}\cdot d^{-1}$.

4.5 CONCLUSIONS

- The obtained results indicate that the SBR is a suitable technology to carry out the autotrophic denitrification of wastewaters containing sulphide and nitrate. Loading rates of 0.45 g NO_3^--$N\cdot L^{-1}\cdot d^{-1}$ and 0.45 g $S^{2-}\cdot L^{-1}\cdot d^{-1}$ were treated with removal efficiencies around 67% and 100%, respectively.

- In order to maintain a full oxidation of sulphide into sulphate and high sulphide removal efficiencies an appropriate control of the pH value inside the system around 7.5-8.0 is needed.

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Chapter 5.

Post-treatment of Fish Canning Effluents by Sequential Nitrification and Autotrophic Denitrification Processes

Abstract

In this chapter a nitrifying/autotrophic denitrifying system was used for the post-treatment of an effluent coming from an anaerobic digester treating the wastewater produced in a fish canning industry. The initial nitrifying reactor was able to fully oxidize ammonia into nitrate with efficiencies around 100%. The effluent from the nitrifying stage was fed to the second autotrophic denitrifying reactor which treated a maximum sulphide loading rate (SLR) of 200 mg S^{2-} L^{-1} d^{-1}. This reactor maintained a removal efficiency of 100% for sulphide while only around 30% of the inlet nitrate was removed. In the first operational stages, this low obtained nitrate removal efficiency can be attributed to sulphide limitations while, during the last operational stage, the decrease of the specific sulphide oxidizing activity caused that the biomass capacity was main limitation.

The estimated cost of operation of this system of 0.80 €/Kg $N_{removed}$, is lower than that calculated for conventional nitrification/denitrification processes but the application of SHARON/anammox processes is the cheapest option to treat this kind of effluents. However the combination of nitrification and autotrophic denitrification (using elemental sulphur) processes would present a better operational stability compared to the SHARON/anammox system.

5.1 INTRODUCTION

Discharge of effluents produced in the fish-canning industry contributes significantly to the contamination of the environment in the littoral zones of the region of Galicia (Spain) (García-Sandá *et al.* 2004). These effluents have a salinity similar to sea water (up to 19 g $Cl^{-}\cdot L^{-1}$, 12 g $Na^{+}\cdot L^{-1}$ and 2.5 g $SO_4^{=}\cdot L^{-1}$), high organic matter content (10-60 g $COD\cdot L^{-1}$), and high protein concentrations (3.5–15.0 $g\cdot L^{-1}$). Anaerobic digestion of these wastewaters achieves COD removal percentages around 70-90%, leading to the formation of high levels of ammonium (up to 5 $g\cdot N\ L^{-1}$) due to protein degradation, producing effluents with a low C/N ratios (Kleerebezem and Méndez 2002; Maya-Altamira *et al.* 2008; Aspé *et al*. 1997; Lozano *et al.* 2003).

The post-treatment of these effluents by conventional nitrification-denitrification processes is not economically feasible since an additional carbon source is needed. Therefore nitrogen removal by autotrophic processes such as nitrification- autotrophic denitrification or nitrification-anammox is an advisable alternative.

The effluent of an anaerobic digester fed with fish canning wastewater has been successfully treated in a combined SHARON-anammox system (Mosquera-Corral *et al*. 2005 Dapena-Mora *et al*., 2006) which allowed the removal of nitrogen loads up to 0.45 g $N\cdot L^{-1}\cdot d^{-1}$ (taking into account the total volume of the needed system) with an average N removal efficiency of 67%. In order to operate the system under stable conditions, the Anammox reactor was fed with an effluent characterized by a NO_2^{-}-N/NH_4^{+}-N ratio lower than 1 g g^{-1}.

High salts concentrations contained in the wastewater from the fish canning industry are known to have negative effects on both organic matter and nitrogen removal processes. The values of EC_{50} (compound concentration which provokes 50% of activity inhibition) reported for methanogenic, nitrifying and anammox biomasses are 20.8, 30.0 and 30.0 g $NaCl\cdot L^{-1}$, respectively (Aspé *et al.*, 1997). Nevertheless, results obtained by several authors showed that autotrophic denitrifying biomass was able to operate under salt concentrations up to 33 g $NaCl\cdot L^{-1}$ without negative effects on its efficiency (Gu *et al.*, 2004; Claus and Kutzner 1985; Campos *et al*., 2008; Koenig and Liu 2004).

Fish cannery wastewater also contains high sulphate concentration. Sulphate is reduced into hydrogen sulphide during anaerobic digestion which must be removed from

the biogas for its utilization for energy generation. Kleerebezem and Méndez (2002) proposed to separate H_2S from methane by absorption in a liquid phase and then to use it as electron donor to remove nitrate in a post-denitrification step. Therefore, combination of nitrification and autotrophic denitrification seems to be a suitable option to remove nitrogen and sulphide compounds from the effluent of an anaerobic digester operating in a fish cannery.

5.2 OBJECTIVE

The objective of this work is to study the operation of a sequential system comprising the nitrification and autotrophic denitrification processes to remove nitrogen from the effluent of an anaerobic digester treating the wastewater from a fish canning industry. Sulphide will be used as electron donor to reduce the nitrate generated during the nitrification step.

5.3 MATERIALS AND METHODS

5.3.1 Reactors

5.3.1.1 Nitrifying Reactor

An activated sludge unit consisting of a mixing basin with a useful volume of 0.5 L coupled to an external settler of 1.0 L was operated during 185 days (Figure 5.1). The system was operated at a hydraulic retention time (HRT) of 1 day and at room temperature (25.0 ± 0.5 ºC). In the aeration basin the pH value ranged from 7.5 to 8.5 and the dissolved oxygen concentration was higher than 2 mg $O_2 \cdot L^{-1}$. A peristaltic pump was used to feed the reactor and a mammoth pump to recirculate the sludge from the settler to the aeration basin. The system was inoculated with activated sludge (1.52 g $VSS \cdot L^{-1}$) collected from the aerobic reactor in operation in a municipal wastewater treatment plant placed in Calo-Milladoiro (A Coruña, Spain).

The reactor was fed with the effluent from an anaerobic digester treating the wastewater of a fish canning industry placed in O Grove, Pontevedra (Spain) (Table 5.1).

As the NH_4^+-N/IC ratio was of 1.09 (g NH_4^+-N g C^{-1}), 1.6 g·L^{-1} of $NaHCO_3$ were added to avoid alkalinity limitation during nitrification.

Table 5.1 Characteristics of the effluent applied to the nitrifying reactor.

Parameter	value	Parameter	value
pH	7.87± 0.26	Conductivity (µs·cm^{-1})	9.5 ± 1.0
COD_T (mg O_2·L^{-1})	914 ± 291	Cl^- (mg·L^{-1})	3800 ± 534
COD_S (mg O_2·L^{-1})	305 ± 83	Br^- (mg·L^{-1})	8.8 ± 1.3
NH_4^+-N (mg·L^{-1})	324 ± 36	SO_4^{-2} (mg·L^{-1})	74 ± 6.6
TC (mg·L^{-1})	357 ± 77	PO_4^{-3} (mg·L^{-1})	32 ± 17.5
IC (mg·L^{-1})	295 ± 70	TSS (mg·L^{-1})	400 ± 21.0
TOC (mg·L^{-1})	62 ± 8	VSS (mg·L^{-1})	295 ± 16.0

Figure 5.1 Nitrifying Reactor

The reactor was operated at a constant HRT of 1 d and the ammonia loading rate (ALR) was gradually increased by decreasing the dilution applied to the effluent collected from anaerobic digester (Table 5.2).

Table 5.2 Operational strategy of the nitrifying reactor.

Stage	Period (d)	Dilution ratio Wastewater:dilution water	Ammonia inlet concentration (mg NH_4^+–N·L^{-1})	Ammonia loading rate (mg N·L^{-1}·d^{-1})
I	0 - 21	1:3	102 ± 7.4	102
II	22 - 46	1:2	175 ± 9.4	175
III	47 - 67	1:1.6	228 ± 15.6	228
IV	68 - 185	no dilution	314 ± 31.0	314

5.3.1.2 Denitrifying Autotrophic Reactor

The autotrophic denitrification was carried out in a sequencing batch reactor (SBR) with a working volume of 1 L (described in Chapter 4). The temperature of operation was maintained at 30 ± 1 ºC by means of a thermostatic jacket. Complete mixture inside the reactor was achieved with a mechanical stirrer operated at 150 rpm. The pH was maintained at 7.5 by controlled addition of acid or base solutions (HCl 0.5 M, NaOH 0.5 M). The HRT was fixed at 1 day. The head space of the reactor was flushed with a mixture of 95% Ar and 5% CO_2 to maintain anoxic conditions (Figure 5.2).

The SBR was operated in cycles of 6 h controlled by means of a PLC (CPU224, Siemens). The operational cycle comprised four phases: feeding in stirring conditions (300 min), stirring (30 min), settling (15 min) and effluent withdrawal (15 min). A volumetric exchange ratio of 25% was applied.

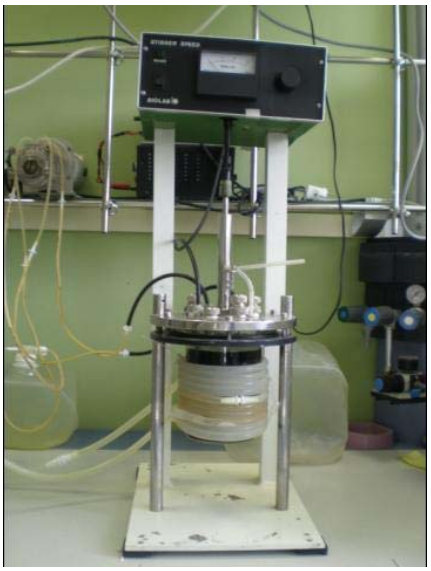

Figure 5.2 Autotrophic denitrifying reactor.

The reactor was operated during 227 days in four operational stages (Table 5.3). In Stage I the reactor was fed with a mineral medium consisting in a mixture of two solutions (A and B) prepared separately in order to avoid the precipitation of metals sulphides. Solution A consisted of ($g \cdot L^{-1}$): $NaHCO_3$ (3.0) and sulphide supplied as $Na_2S \cdot 3H_2O$ (1.27); with pH value adjusted to 8.0 by addition of HCL 1 M. Solution B consisted of ($g \cdot L^{-1}$): $NaNO_3$ (5.46); Na_2HPO_4 (3.0); KH_2PO_4 (3.6); NH_4Cl (0.2); $MgSO_4$ anhydrous (0.08); and 2 mL per litre of a traces solution. The traces solution contained ($g \cdot L^{-1}$): $Na_2MoO_4 \cdot 2H_2O$ (1.0); $FeSO_4 \cdot 7H_2O$ (38.0); $CaCO_3$ (2.0); $ZnSO_4 \cdot 7H_2O$ (1.5); $MnCl_2 \cdot 4H_2O$ (1.0); $CuSO_4 \cdot 5H_2O$ (0.25); $CoCl_2 \cdot 6H_2O$ (0.25); $NiCl_2 \cdot 6H_2O$ (0.25); H_3BO_3 (0.5); and HCl (50.0 $g \cdot L^{-1}$). Solutions A and B were fed to the reactor separately at the same flow rate (0.5 $L \cdot d^{-1}$). The final nitrate and sulphide concentrations were fixed at 142 mg $S^{2-} \cdot L^{-1}$ and 453 mg NO_3^--$N \cdot L^{-1}$ and the S^{2-}/NO_3^- ratio was of 0.13 ($mol \cdot mol^{-1}$).

During Stage II Solution B was replaced by the effluent of the nitrifying reactor treating the fish canning industry effluent without dilution (corresponded to Stage IV of the

nitrifying reactor). The inlet nitrogen concentration was of 149 mg NO_3^--N·L^{-1} and the applied S^{2-}/NO_3^- ratio of 0.45 (mol·mol^{-1}).

In the Stage III with the aim to increase the applied NLR the flow rate of Solution B was increased up to 0.8 L·d^{-1} while the flow rate of Solution A was decreased down to 0.2 L·d^{-1} to maintain the HRT at 1 d. In order to maintain the inlet sulphide and $NaHCO_3$ concentrations, both were increased in solution A up to 4.12 g $Na_2S·3H_2O·L^{-1}$ and 7.5 g $NaHCO_3$ ·L^{-1}, respectively. By using this strategy the inlet nitrogen concentration was of 262 mg NO_3^--N·L^{-1} and the applied S^{2-}/NO_3^- ratio of 0.28 (mol·mol^{-1}).

During Stage IV sulphide concentration was increased up to 5.15 g $Na_2S·3H_2O·L^{-1}$ in Solution A to improve nitrate removal efficiency. The inlet sulphide and nitrate concentrations were 203 mg S^{2-}·L^{-1} and 218 mg NO_3^--N·L^{-1}, respectively, and the S^{2-}/NO_3^- ratio applied was of 0.41 (mol·mol^{-1}).

Table 5.3 Operational conditions of the autotrophic denitrifying reactor.

Stage	Feeding medium	Stage (d)	NLR (g N·L^{-1}·d^{-1})	SLR (mg S^{2-}·L^{-1}·d^{-1})	S^{-2}/NO_3^- ratio (mol·mol^{-1})
I	Mineral medium	0 - 121	466 ± 26	142 ± 17	0.13
II	Nitrified effluent	122 - 181	149 ± 22	156 ± 13	0.45
III	Nitrified effluent	182 - 209	262 ± 13	168 ± 16	0.28
IV	Nitrified effluent	210 - 227	218 ± 20	203 ± 2.6	0.41

5.3.2 Analytical methods

The pH value and the volatile suspended solids (VSS) concentration were determined according to the Standard Methods (APHA, 1999). Nitrite, nitrate and sulphate concentrations were determined by ion chromatography while sulphide and dissolved oxygen ones were measured by means of ion-selective electrodes (see Chapter 2).

Ammonium oxidizing and autotrophic denitrifying activities were estimated according to the methodology described in Chapter 2.

5.3.3 Calculations

The efficiency of the nitrifying reactor was calculated according to equation 5.1:

$$\%NH_4^+ - N_{removal} = \frac{\left[NH_4^+ - N\right]_i - \left[NH_4^+ - N\right]_e}{\left[NH_4^+ - N\right]_i} \cdot 100 \qquad [5.1]$$

Where:

$[NH_4^+\text{-}N]_i$ = ammonia N concentration in the influent (mg $N \cdot L^{-1}$)

$[NH_4^+\text{-}N]_e$ = ammonia N concentration in the effluent (mg $N \cdot L^{-1}$)

The specific Ammonia Loading Rate (ALR) removed (mg NH_4^+-$N \cdot g\ VSS^{-1} \cdot d^{-1}$) in the nitrifying reactor was calculated according equation 5.2:

$$Specific\ ALR_{removed} = \frac{\left[NH_4^+ - N\right]_i - \left[NH_4^+ - N\right]_e}{HRT \cdot X} \qquad [5.2]$$

Where:

HRT= Hydraulic retention time (d)

X= biomass concentration (g VSS·L^{-1})

The nitrogen removal efficiency of the denitrifying unit was calculated according to equation 5.3:

$$\%N_{removal} = \frac{\left[NO_3^- - N\right]_i - \left[NO_3^- - N\right]_e - \left[NO_2^- - N\right]_e}{\left[NO_3^- - N\right]_i} \cdot 100 \qquad [5.3]$$

Where:

$[NO_3^-\text{-}N]_i$ = nitrate N concentration in the influent of the denitrifying system (mg N·L^{-1})

$[NO_3^-\text{-}N]_e$ = nitrate N concentration in the effluent of the denitrifying system (mg N·L^{-1})

$[NO_3^-\text{-}N]_e$ = nitrite N concentration in the effluent of the denitrifying system (mg N·L^{-1})

The sulphide removal efficiency of the denitrifying reactor was calculated taken into account the inlet and outlet sulfide concentrations:

$$\%S^{2-}_{removal} = \frac{\left[S^{2-}\right]_i - \left[S^{2-}\right]_e}{\left[S^{2-}\right]_i} \cdot 100 \qquad [5.4]$$

Where:

$[S^{2-}]_i$ = sulphide concentration in the influent of the denitrifying reactor (mg S·L^{-1})

$[S^{2-}]_e$ = sulphide concentration in the effluent of the denitrifying reactor (mg S·L^{-1})

The average sludge retention time (SRT) of both reactors was calculated for the different operational stages according to equation 5.5:

$$SRT = \frac{VSS_R \cdot V_R}{VSS_e \cdot Q} \quad [5.5]$$

Where:

SRT= suldge retention time (d)

VSS_R= volatile suspended solids concentration in the reactor ($g \cdot L^{-1}$)

VSS_e= volatile suspended solids concentration in the effluent ($g \cdot L^{-1}$)

V_R=volume of the reactor (L)

Q= Inflow rate ($L \cdot d^{-1}$)

5.4 RESULTS

5.4.1 Operation of the nitrifying reactor

The nitrifying reactor was operated during 185 days. The ammonia removal efficiency ranged between 93 and 95% during Stages I, II and IV (Table 5.4). This efficiency decreased down to 88% during Stage III due to the decrease of the pH value inside the system to values around 6. During the whole operational period the final product was always nitrate while nitrite was not detected even during Stage III (Figure 5.3). During the four operational stages the applied ALR was gradually increased from 102 to 314 mg NH_4-$N \cdot L^{-1} \cdot d^{-1}$ (Figure 5.4).

Table 5.4 Performance of the nitrifying reactor.

Stage	NH_4^+-N influent ($mg \cdot L^{-1}$)	NH_4^+-N effluent ($mg \cdot L^{-1}$)	NO_3^--N effluent ($mg \cdot L^{-1}$)	NH_4^+-N removal (%)
I	102 ± 7.4	6.9 ± 2.5	97.1 ± 6.2	93.1
II	175 ± 9.4	8.4 ± 3.0	157 ± 18.1	95.2
III	228 ± 15.6	27.4 ± 17.3	175 ±17.0	88.0
IV	314 ± 31.0	15.6 ± 3.0	300 ± 40.3	95.0

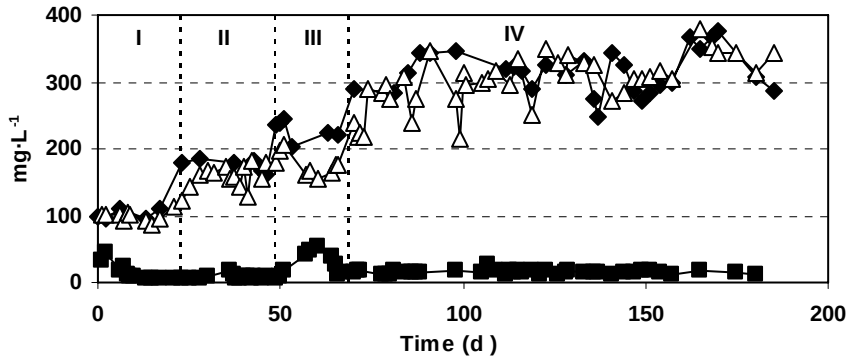

Figure 5.3 Nitrifying reactor: concentrations of NH_4^+-N influent (♦), NH_4^+-N effluent (■) and NO_3^--N effluent (Δ); ($mg \cdot L^{-1}$).

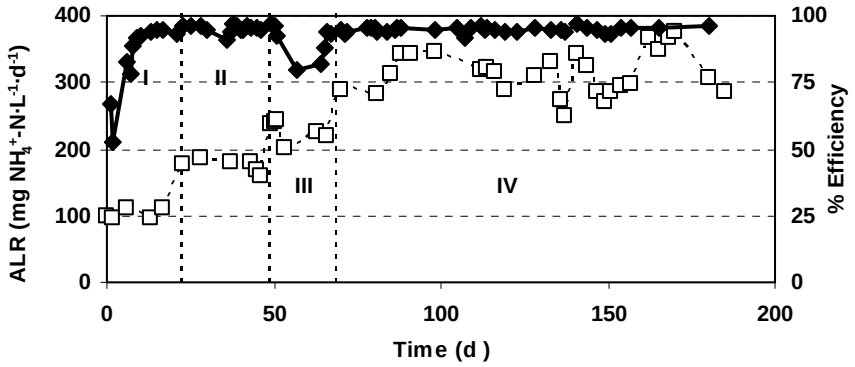

Figure 5.4 Nitrifying reactor: applied ALR (□) (mg NH_4-N·L^{-1}·d^{-1}) and ammonia oxidation efficiency (♦) (%).

5.4.1.1 Evolution of the biomass concentration and the SRT

After inoculation and during Stage I, the biomass concentration decreased from 1.45 to 0.68 g VSS·L^{-1} (Figure 5.5). In this period the average VSS concentration in the effluent was 25 mg VSS·L^{-1} which involves a SRT value of 6.8 d (Figure 5.6). During Stages II and III both biomass concentrations in the reactor and in the effluent remained practically constant at values of VSS of 0.8 g VSS·L^{-1} and 20 mg VSS·L^{-1}, respectively. The SRT value was at this point of 10 days. When the inlet ammonium concentration was increased up to 314 mg NH_4-N·L^{-1} (Stage IV) the biomass concentration increased significantly up to 1.8 g VSS·L^{-1} while the biomass concentration in the effluent remained practically constant at values around of 20 mg VSS·L^{-1}. This caused an increase of the SRT up to 24 days. During whole operational period the ashes content in the sludge represent a 33% of the TSS content. This value is in the range of 16-35% previously reported by Mosquera-Corral *et al.* (2005) for nitrifying biomass treating an effluent with similar composition in a partial nitrifying reactor.

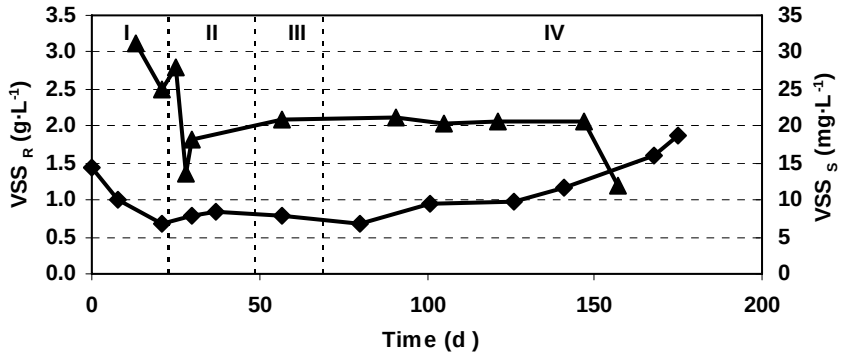

Figure 5.5 Concentrations of VSS in the reactor (♦) and the effluent (▲); ($g \cdot L^{-1}$).

5.4.1.2 Ammonium oxidizing activity

In order to estimate of the maximum specific nitrifying activity (SNA) of the biomass, batch assays were performed with biomass collected during different operational periods (Table 5.5). In the presence of 1.2 g $Cl^{-} \cdot L^{-1}$ (Stage I), the maximum estimated SNA was of 33 mg NH_4^{+}-N·g $VSS^{-1} \cdot d^{-1}$ while when the reactor was operated at chloride concentrations of 2.0 g $Cl^{-} \cdot L^{-1}$ (Stage II) the maximum measured SNA increased up to 98 mg NH_4^{+}-N·g $VSS^{-1} \cdot d^{-1}$. This stimulation of the nitrifying activity due to the increase of salt concentration was already reported by Mosquera-Corral *et al*. (2005). These authors observed an increase of 26% of the nitrifying activity when the chloride concentration was increased from 0 to 3 g $Cl^{-} \cdot L^{-1}$. A similar tendency was also found for anammox microorganisms (Kartal *et al*., 2006; Dapena-Mora *et al*., 2010). Several authors reported that after an adaptation period to high salinity concentrations the microbial activity might be favoured by a possible selection of micro-organisms resistant to these operational conditions (Chen *et al*., 2003). However this positive effect was also reported with non-adapted biomass in the case of anammox micro-organisms (Dapena-Mora *et al*., 2010). On day 175 of operation (Stage IV) when the system was operated at 3.8 g $Cl^{-} \cdot L^{-1}$ (Stage IV), the maximum SNA decreased down to 40 mg NH_4^{+}-N·g $VSS^{-1} \cdot d^{-1}$.

Table 5.5 Nitrifying activities

Operation day	Cl^- ($g \cdot L^{-1}$)	Maximum SNA (mg NH_4^+-N·g $VSS^{-1} \cdot d^{-1}$)
17	1.2	33
37	2.0	98
175	3.8	40

The values of the specific applied ALR, calculated during the operational period, ranged from 75 to 418 mg NH_4^+-N·g VSS^{-1} d^{-1} (Figure 5.6). Such values obtained during the reactor operational period are significantly higher than those corresponding to the maximum SNA obtained from the batch tests. This fact was also observed by Dapena-Mora *et al*. (2010) in the case of anammox biomass who found that in those batch activity assays performed under salinity conditions different than those existing inside the reactor the obtained specific activity values could not explain the efficiency observed inside the reactor. Only reliable values were obtained when the batch assays were performed under the same salinity conditions as those present inside the reactor. This fact could be attributed to changes in the osmotic pressure which affect the movement of substrates across the bacteria cell membrane and, hence, their activity (Koenig and Liu, 2004).

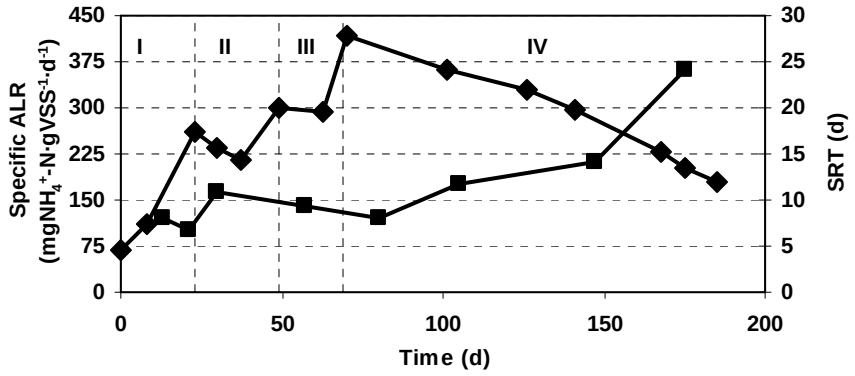

Figure 5.6 Specific ALR applied (♦) (mg NH_4^+-N·g $VSS^{-1} \cdot d^{-1}$) and SRT (■) (d).

5.4.2 Autotrophic denitrifying reactor

During the whole operational period, which lasted 227 days, the reactor was able to remove the applied SLR with efficiencies of 100% (Table 5.6). All sulphide was practically recovered as sulphate and elemental sulphur formation was not detected (Figure 5.7). In this work, the influent had a S^{2-}/NO_3^- ratio ($mol \cdot mol^{-1}$) lower than the stoichiometric one and the reactor was operated at pH values around the neutrality which avoided the possible accumulation of elemental sulphur (Kleerebezem and Méndez 2002).

Table 5.6 Performance of the autotrophic denitrifying reactor.

Stage	NO_3^--N influent (mg L^{-1})	S^{2-} influent (mg L^{-1})	NO_3^--N effluent (mg L^{-1})	NO_2^--N effluent (mg L^{-1})	S^{2-} effluent (mg L^{-1})	SO_4^{2-}-S effluent (mg L^{-1})	Sulphide removed (%)	Nitrogen removed (%)
I	453 ± 52.4	142 ± 17.0	362 ± 37.2	32 ± 22.3	0	110 ± 20.5	100	13.0
II	154 ± 23.5	156± 13.6	80.3 ± 12.0	23.4 ± 10.4	0	157 ± 12.0	100	32.6
III	262 ± 13.8	168 ± 21.2	107 ± 12.7	69.0 ± 2.8	0	143 ± 12.1	100	32.8
IV	204 ± 16.7	202 ± 2.6	66.8± 8.0	75 ± 7.7	0	204 ± 13.8	100	30.3

No negative effects on the sulphide removal efficiency were observed when the synthetic feeding media was changed by the effluent of the nitrifying reactor (Stage II). During Stage IV, the system was able to treat a SLR of 200 mg $S^{2-}\cdot L^{-1}\cdot d^{-1}$ (Figure 5.7).

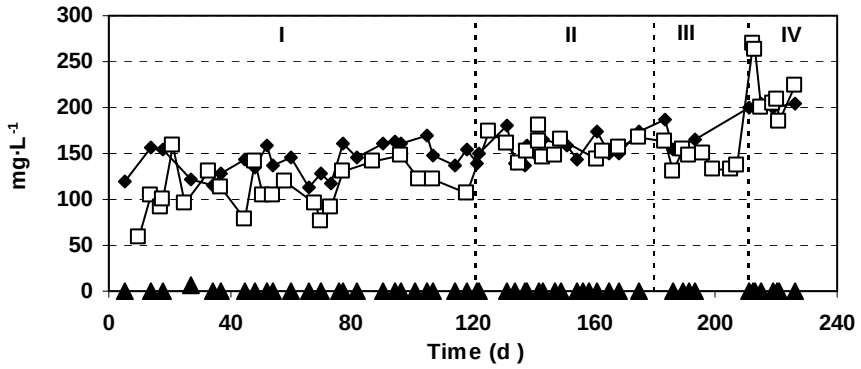

Figure 5.7 Sulphur compound: S^{2-} influent (♦), S^{2-} effluent (▲) and SO_4^{2-}-S effluent (□); ($mg\cdot L^{-1}$).

The reactor was always operated under sulphide limiting conditions in order to prevent possible inhibitory effects of this compound. This caused both, the incomplete nitrate removal efficiency and the apparition of nitrite as an intermediate product (Figure 5.8). Therefore, the nitrate removal efficiency was directly related to the S^{2-}/NO_3^- ratio of the feeding medium (Figure 5.9).

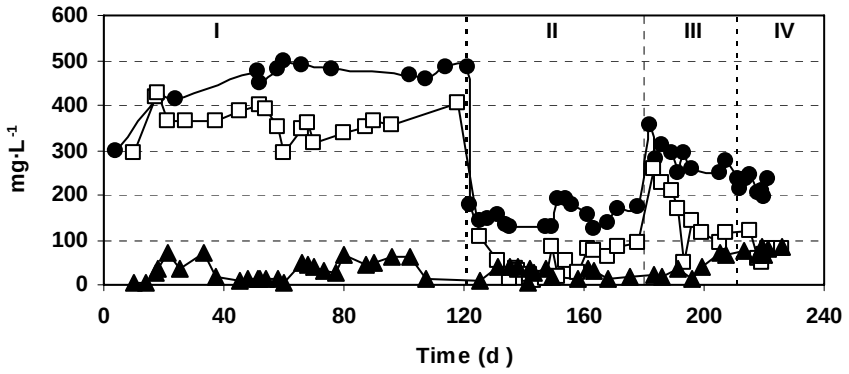

Figure 5.8 Nitrogen compounds concentrations NO_3^--N influent (♦), NO_3^--N (□) and NO_2^--N effluent (▲); ($mg \cdot L^{-1}$)

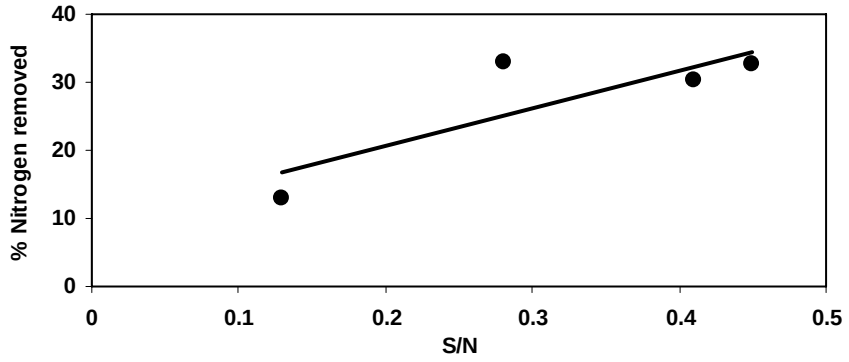

Figure 5.9 Percentage of nitrogen removed at different applied S^{2-}/NO_3^- ratios.

During the reactor operation the removed nitrogen loading rate was increased up to values of 75 mg NO_3-$N \cdot L^{-1} \cdot d^{-1}$ with the simultaneous and proportional increase of produced nitrite, which indicates incomplete denitrification occurrence due to sulphide limitation (Figure 5.10). A similar behaviour was found during operation of the reactor in chapter 4 of this thesis (Figure 4.7).

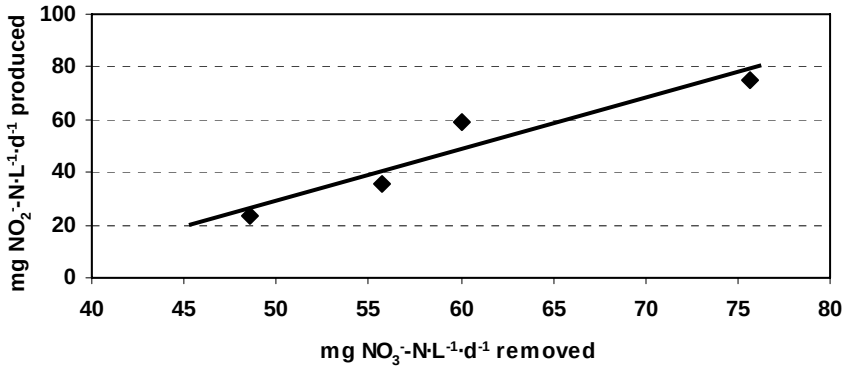

Figure 5.10 Nitrite produced in correlation to the nitrate removed.

5.4.2.1 Activity measurements

During Stage I, when the reactor was fed with the synthetic medium, the maximum specific sulphide oxidizing activity was of 219 mg S^{2-}·g VSS^{-1}·d^{-1} (Figure 5.11). However, during Stage II when the nitrified effluent was applied, the maximum specific sulphide oxidizing activity decreased down to 131 mg S^{2-}·g VSS^{-1}·d^{-1}. The maximum specific activity gradually decreased during Stages III and IV when the proportion of nitrified effluent was increased. The value of the maximum specific sulphide oxidizing activity was of 45.6 mg S^{2-}·g VSS^{-1}·d^{-1} during the last stage which indicates a decrease of 78% with respect to the initial value. Despite this fact the efficiency of sulphide removal in the reactor was of 100% (Figure 5.7), since the specific sulphide loading rate applied was always lower than the maximum specific sulphide oxidizing activity (Figure 5.11).

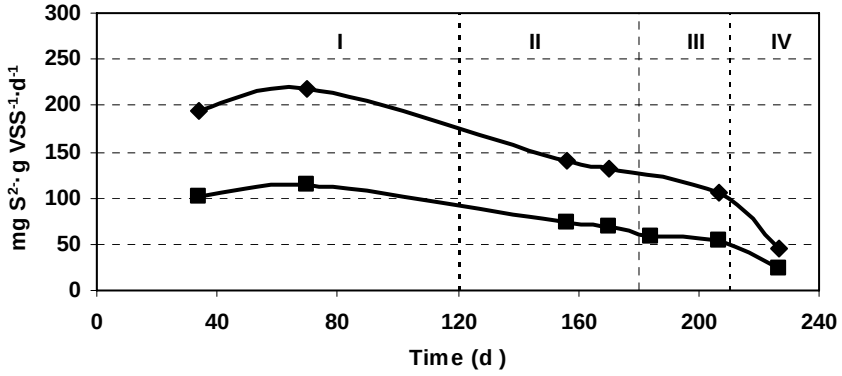

Figure 5.11 Specific sulphide oxidizing activity (♦) and specific applied SLR (■); (mg S^{2-}·g VSS^{-1}·d^{-1})

The possible reasons for the observed loss of the specific biomass activity are analyzed in the following section:

a) The increase of the salinity. The average chloride concentration of the nitrified effluent was 3.8 g Cl^-·L^{-1}. Taken into account the dilution applied to this effluent, the maximum chloride concentration expected inside the reactor would be around 3.0 ± 0.07 g Cl^-·L^{-1}. It is widely reported the absence of negative effects on the autotrophic denitrification of concentrations up to 12 g Cl^-·L^{-1} (Claus and Kutzner, 1985; Koenig and Liu, 2004; Campos *et al*., 2008). Therefore, the loss of the specific activity could not be attributed to the presence of chloride.

b) Presence of nitrite. Nitrite has a strong inhibitory effect on autotrophic denitrifying biomass (Krishnakumar and Manilal, 1999). Nevertheless, the highest nitrite concentrations were observed during Stage I when the maximum specific sulphide oxidizing activities were the highest ones and, therefore, there is not clear relation between nitrite concentration inside the system and the variation of the specific activity.

Both biomass concentrations in the reactor and effluent remained almost constant at 3.6 g VSS·L^{-1} and 20 mg VSS·L^{-1}, respectively, during the operational period (Figure 5.12) and the SRT was of 186 d. During the whole operational period the ashes content in the sludge represented a 30% of the TSS.

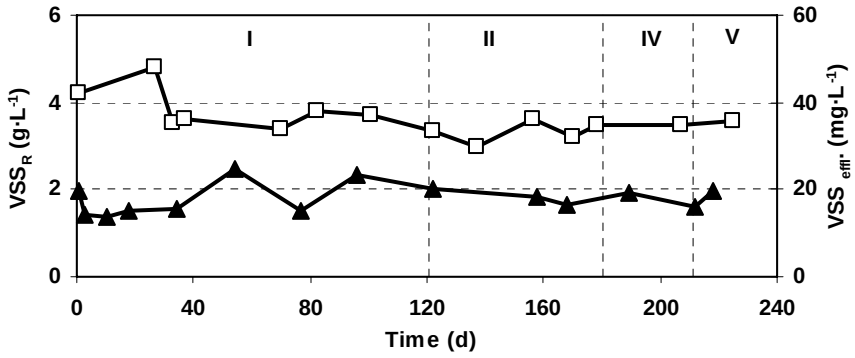

Figure 5.12 Concentrations of VSS (□) in the reactor and in the effluent (▲); (mg·L^{-1}).

5.4.2.2 Overall system performance

Both nitrifying and denitrifying reactors maintained a stable operation when they were fed with the effluent coming from the anaerobic digester of a fish cannery. The nitrifying reactor treated an ALR around 0.3 g NH_4^+-N·L^{-1}·d^{-1} but the autotrophic denitrifying system was only able to remove a maximum nitrate loading rate (NLR) of around 0.1 g NO_3^--N·L^{-1}·d^{-1}. During Stages I to III this low removal rate was due to the low applied S^{2-}/NO_3^- ratio to the system in order to avoid possible inhibitory effects of sulphide. However, at the end of Stage IV the specific applied SLR was similar to the specific sulphide oxidizing activity which indicated that finally the biomass capacity was the limiting factor (Figure 5.11).

According to the literature research works have been performed where nitrogen was removed from effluents coming from anaerobic digesters treating fish canning industries

wastewater treated by using different biological processes. The applied NLR and the achieved nitrogen removal efficiencies ranged from 0.02 to 0.45 g $N \cdot L^{-1} \cdot d^{-1}$ and from 20 to 80%, respectively (Table 5.7). When the nitrification/denitrification processes were used, the nitrogen removal efficiency was limited by the low amount of available organic matter to carry out the denitrification process complete. During the application of anammox and autotrophic denitrification processes, full nitrogen removal efficiency was not achieved because both systems were under nitrite and sulphide limiting conditions, respectively, in order to avoid possible inhibitory effects of these compounds.

Table 5.7. Processes applied for the treatment of effluents from anaerobic digesters treating fish canning industry wastewater.

Process	NLR ($g\ N \cdot L^{-1} \cdot d^{-1}$)	Nitrogen removal (%)	Reference
Nitrification/Denitrification	0.16 - 0.28	20 – 45	Figueroa *et al.* (2008)
Partial nitrification/Anammox	0.20 – 0.45*	40 – 80	Vázquez-Padín *et al*. (2009)
Nitrification/Autotrophic denitrification	0.02 – 0.08*	13 – 33	This study

*Taking into account the total volume of the needed system.

Previous works indicate that removed NLRs in autotrophic denitrifying systems using sulphide as sulphur source ranged between 0.1 and 0.6 g NO_3^-- $N \cdot L^{-1} \cdot d^{-1}$ (Manconi *et al*., 2006; Wang *et al.*, 2005; Vaiopoulou *et al*., 2005; Gadekar *et al*., 2006) while NLRs up to 2 g NO_3^--$N \cdot L^{-1} \cdot d^{-1}$ can be removed when Sº is used as sulphur source. Moreover when sulphide is used, the low applied S^{2-}/NO_3^- ratio causes the formation of nitrite as an intermediate product, which decreases both the nitrogen removal efficiency and the quality of the produced effluent. Nevertheless, when autotrophic denitrification is carried out with Sº no control of the S^{2-}/NO_3^- ratio is required to avoid the formation of nitrite. Therefore the use of elemental sulphur instead of sulphide could be an interesting option to improve the performance of the autotrophic denitrifying reactors. The H_2S coming from biogas could be previously oxidized under microaerobic conditions into Sº and then, under anoxic conditions, into sulphate using nitrate as electron acceptor.

The S^{2-}/NO_3^- ratio of the fish canning effluents ranged from 0.83 to 1.5 g S·g N^{-1} (Aspé *et al*.,1997; Dapena-Mora *et al*., 2006; Veiga *et al*., 1994; Kleerebem and Méndez 2000). Therefore, in most cases sulphide present in the biogas is not enough to remove all the nitrogen from the wastewater and Sº addition to the denitrifying system is required. Nevertheless this process is still competitive under an economical point of view compared to the conventional nitrification/denitrification processes to treat this kind of effluents (Table 5.8).

Table 5.8 Estimation of the operational costs to remove 1 kg of nitrogen using different processes (adapted from Fernández, 2010).

Process	Electricity (€)	Substrate (€)	Sludge (€)	Total cost (€)	Reference
Nitrification/Autotrophic denitrification with Sº	0.39	0.25*	0.16**	0.80	Park and Yoo (2009)
Partial nitrification/Autotrophic denitrification with Sº	0.31	0.15***	0.13	0.59	Park and Yoo (2009)
Conventional N/D + MeOH	0.39	0.75	0.27	1.41	Fernández (2010)
SHARON/anammox	0.18	-----	0.04	0.22	Fernández (2010)

*0.1 €·kg S° and 2.5 kg Sº consumed· (kg NO_3^--N)$^{-1}$

** An average yield coefficient of 0.5 g VSS·(g NO_3^--N) $^{-1}$ was used for autotrophic denitrifying bacteria

***1.5 kg Sº consumed·(kg NO_3^--N)$^{-1}$

The combination of SHARON (partial nitrification) and anammox processes is the cheapest option to remove nitrogen from the effluents of anaerobic digesters from fish canneries even if it is compared to the combination of partial nitrification and autotrophic denitrification with Sº (Table 5.8). The main advantage of the application of the SHARON/anammox processes is related to the fact that only around 60% of ammonia is oxidized into nitrite which mainly decreases the aeration costs. Nevertheless, the post-treatment of fish canneries effluents by SHARON-anammox processes can present some

disadvantages due to the difficulty to maintain a stable performance of the SHARON system. The presence of organic matter in the effluent can cause fluctuations in the efficiency of the SHARON unit and an optimal nitrite to ammonium ratio is not easily achieved in this system to feed the anammox reactor (Vázquez-Padín *et al.,* 2009). On the other hand, the presence of sulphide contained in the effluent produced in these anaerobic digesters could cause the loss of the nitrification activity (Beristain-Cardoso *et al.*, 2010). To prevent this effect the presence of a previous aerobic unit in order to oxidize the sulphide is recommended. However the implementation of this new unit increases both capital investment and operational costs. In this case, the application of a nitrifying system followed by an autotrophic denitrifying system can be more stable in terms of nitrogen removal efficiency. Since in this system ammonia is fully oxidized into nitrate, the nitrifying unit can be operated with high biomass content which makes it more resistant to the presence of organic matter and sulphide. Moreover, this system can be used also to remove sulphide from biogas with a consequent reduction of operational costs related to the biogas cleaning for its further use for energy production (Deng *et al.*, 2009; Ramírez-Sáenz *et al.*, 2009; Chaiprapat *et al.*, 2011).

5.5. CONCLUSIONS

- The nitrifying reactor was able to treat an ALR around 0.3 g NH_4^+-N·L^{-1}·d^{-1} with an efficiency close to 100% when it was fed with the effluent of an anaerobic digester of a fish cannery.

- The autotrophic denitrifying system was operated under limiting sulphide conditions, to avoid the possible negative effects of this compound, which caused that the nitrogen removal efficiency was only of 30%. The maximum specific sulphide oxidizing activity decreased along the operational period and, finally, the NLR treated by the system was limited by the biomass capacity.

- For the post-treatment of fish canneries effluents, the operational costs of the nitrifying/autotrophic denitrifying system using elemental sulphur as electron donor are lower than those of the conventional nitrification/denitrification processes. The lowest

operational costs are obtained when the SHARON/anammox processes are applied but the nitrifying/autotrophic denitrifying system is more stable in terms of nitrogen removal efficiency.

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Chapter 6

Kinetics aspects on autotrophic denitrification process with sulphide as electron donor

Summary

In this chapter the kinetic parameters of autotrophic denitrifying biomass using sulphide as electron donor were determined.

The autotrophic denitrifying biomass was inhibited at nitrate concentrations over 200 mg NO_3^--N·L^{-1}, the Haldane´s model was the model that best explained this inhibition. The next parameters were determined: V_{max}= 0.470 ± 0.0013 mg N_2·L^{-1}·min^{-1}, Ks= 13.51 ± 0.113 mg L^{-1} and K*i*=355 ± 4.38 mg L^{-1}.

Inhibition by nitrite was also observed and it occurred at concentrations four times lower than those found for nitrate. A value of IC_{50} of 48 mg NO_2^--N·L^{-1} was obtained. Nitrous oxide was detected as product of denitrification, which is the main responsible of inhibition.

Ammonium and phosphate had not inhibitory effect on denitrification at different concentrations tested. More research would be necessary about the effects of these two compounds.

The optimum values of temperature and pH to carry out the complete autotrophic denitrification were 35 ºC and 8.0, respectively.

6.1 INTRODUCTION

The growth of industrial activities has generated serious environmental problems. There are many discharge wastewater containing nitrogen and sulphur compounds. Nitrogen is normally present as ammonium, nitrite or nitrate while sulphur can be both as reduced or oxidised forms (Tandukar *et al*., 2009). Nitrates and sulphides discharges have high environmental impact, the main environmental problem associated to nitrates being acidification of water bodies, eutrophication of aquatic ecosystems and health problems, while the release of sulphides into environment provokes acid rain, unpleasant odours, and even intoxication. For these reasons suitable technologies to remove them from wastewater are necessaries. Autotrophic denitrification process is a good option to remove jointly both contaminants (Krishnakumar *et al.,* 2005; Vaiopoulou *et al*., 2005).

Several researches have been performed to establish optimal conditions for autotrophic denitrification, refered to the kind of sulphur compound, Sulphur/Nitrogen (S/N) ratio, pH, Nitrogen Loading Rate (NLR) and Sulphur Loading Rate (SLR), temperature; accumulation of intermediates, and mass transfer (Krishnakumar and Manilal 1999; Claus and Kutzner 1985; Oh *et al*., 2000; Campos *et al*., 2008).

Despite several researches on autotrophic denitrification using sulphide as electron donor has been developed (Kleerebezem and Méndez 2000; Vaiopoulou *et al*. 2005; Gadekar *et al.* 2006), less attention has been paid to the effects imposed by sulfide concentration on autotrophic denitrification. The sensitivity at different sulphide concentrations depends on several factors such as type of microorganism, pH, temperature, nitrogen compound used as electron acceptor and intermediate compounds (Krishnakumar and Manilal 1999; Tugtas and Pavlostathis 2007). However there are not conclusive results over the effect of sulphide concentration on autotrophic denitrification. Several authors have reported an inhibitory effect of sulphide on the last step of denitrification, reduction of nitrous oxide into nitrogen gas, consequently causing accumulation of nitrite, nitrous oxide and nitric oxide (Sorensen *et al*., 1980; Knowles 1982)

The values of kinetics parameters depend of sulphur compound and type of biomass (Claus and Kutzner 1985; Oh *et al*., 2000; Tandukar *et al.,* 2009). Most of the kinetics parameters have been calculated mainly in systems containing thiosulphate or elemental sulphur as electron donor, while few studies have been done using sulphide. Gadekar *et al*. (2006) reported some kinetics parameters as μ_{max} =0.36 h^{-1}, K_s =1.99 mmol sulphide, k_d = 0.0014 h^{-1}, Y_{S2-}= 0.018 mg ATP/mmol S^{2-} and sulphide removal rate of 2.4 mM/h. More

information on kinetics parameters is necessary for a better understanding of autotrophic denitrification when sulphide is used as electron donor.

6.2 OBJECTIVES

The operation of the autotrophic denitrifying reactor presented in chapter 4 was under sulphide limiting condition their behaviour being influenced by operational conditions as S/N ratio, mineral composition of feeding, pH and intermediate products which affected their efficiency.

Therefore, the overall objective of this chapter was to study the influence of different parameters on autotrophic denitrification with sulphide as electron donor.

The specific objectives of this chapter were to study:

- The influence of sulphide and nitrate concentrations on autotrophic denitrification.
- The influence of ammonium concentration on autotrophic denitrification.
- The possibility to use nitrite as electron acceptor during the autotrophic denitrification of sulphide.
- The influence of temperature on autotrophic denitrification.
- The possible cross-effect of T/pH/NH_4^+ on autotrophic denitrification.
- The influence of phosphate concentrations on autotrophic denitrification.

6.3 MATERIALS AND METHODS

6.3.1 Experimental planning

6.3.1.1 Assays on the effect of the concentration of sulphide and nitrate on the autotrophic denitrification.

In this set of experiments appropriate amounts of two stock solutions of nitrate (A: containing 47.2 g $NaNO_3 \cdot L^{-1}$) and sulphide (B: containing 61.1 g $Na_2S \cdot 3H_2O$ L^{-1} in oxygen-free water) were introduced in tree vials of 37 mL to reach the final concentration presented in Table 6.1. An S/N ratio of 0.84 mol/mol was maintained constant in all experiments.

Table 6.1 Assays to evaluate the effect of the concentration of sulphide and nitrate

Assay	1	2	3	4	5	6	7
S^{2-} (mg·L^{-1})	25	50	75	100	150	200	300
NO_3^--N (mg·L^{-1})	13	26	39	52	78	104	156

6.3.1.2 Assays on the effect of ammonium concentration on the autotrophic denitrification

In this set of experiment appropriate amounts of stock solutions of nitrate (A: containing 47.2 g $NaNO_3$·L^{-1}), sulphide (B: containing 61.1 g $Na_2S·3H_2O·L^{-1}$ in oxygen-free water) and ammonium (C: containing 11.8 g·L^{-1} NH_4Cl) were introduced in three vials of 37 mL to reach the final concentration of 75 as mg S^{2-} $L^{-1}$and 39 mg NO_3^--N L^{-1} respectively for all assays, changing the ammonium concentrations as presented in table 6.2.

Table 6.2 Assays to evaluate the effect of ammonium concentration

Assay	1	2	3	4	5
NH_4^+- N (mg·L^{-1})	10	25	50	75	100

6.3.1.3 Assays on the effect of the concentration of sulphide and nitrite on the autotrophic denitrification

In this set of experiments appropriate amounts of two stock solutions of nitrite (A: containing 81 g $NaNO_2$·L^{-1}) and sulphide (B: containing 61.1 g $Na_2S·3H_2O·L^{-1}$ in oxygen-free water) were introduced in tree vials of 37 mL to reach the final concentrations presented in Table 6.3. A S/N ratio of 0.37 mol/mol was maintained constant in all experiments.

Table 6.3 Assays to evaluate the effect of the concentration of sulphide and nitrite

Assay	1	2	3	4	5	6	7
S^{2-} (mg·L^{-1})	25	50	75	100	150	200	300
NO_2^--N (mg·L^{-1})	29	58	87	116	174	232	348

6.3.1.4 Assays on the effect of temperature on the autotrophic denitrification

In this set of experiments appropriate amounts of two stock solutions of nitrate (A: containing 47.2 g $NaNO_3$·L^{-1}), sulphide (B: containing 61.1 g $Na_2S·3H_2O·L^{-1}$ in oxygen-free

water) where introduced in tree vials of 37 mL to reach the final concentration of 75 mg $S^{2-}\cdot L^{-1}$ and 39 mg NO_3^--N·L^{-1} respectively for all assays. Three temperatures were tested: 25, 30 and 35 ºC. A S/N ratio of 0.84 mol/mol was maintained constant in all experiments.

6.3.1.5 Assays on the cross-effect of T/pH/NH_3^+ on autotrophic denitrification

In this set of experiments appropriate amounts of two stock solutions of nitrate (A: containing 47.2 g $NaNO_3\cdot L^{-1}$) and sulphide (B: containing 61.1 g $Na_2S\cdot 3H_2O\cdot L^{-1}$ in oxygen-free water) were introduced in tree vials of 37 mL to reach the final concentration of 75 mg $S^{2-}\cdot L^{-1}$ and 39 mg NO_3^--N·L^{-1} respectively for all assays. Ranges of temperature, pH and free ammonium concentration are presented in table 6.4. A S/N ratio of 0.84 mol/mol was maintained constant in all experiments.

Table 6.4 Assays to evaluate the cross-effect of T/pH/NH_3^+

Assay	1	2	3	4	5	6	7	8	9
Temperature º C	15 º C			25 º C			35 º C		
pH	7	8	9	7	8	9	7	8	9
NH_3^+ (mg L^{-1})	0.27	2.67	21.3	0.56	5.347	36.2	1.12	10.1	53.1

6.3.1.6 Assays on the effect of phosphate concentrations on the autotrophic denitrification

This experiment was performed with a mineral medium containing (g·L^{-1}): 1.5 $NaHCO_3$, 0.04 $MgSO_4$, 0.1 NH_4Cl and 1 mL of micronutrients (see Chapter 3) and appropriate amounts of stock solutions of nitrate (A: containing 47.2 g $NaNO_3\cdot L^{-1}$), sulphide (B: containing 61.1 g $Na_2S\cdot 3H_2O\cdot L^{-1}$ in oxygen-free water) and phosphate (C: containing 74.8 g $Na_2HPO_4\cdot L^{-1}$ and 90 g $KH_2PO_4\cdot L^{-1}$). They were introduced in tree vials of 37 mL to reach the final concentrations of 75 mg $S^{2-}\cdot L^{-1}$and 39 mg NO_3^-N·L^{-1} respectively for all assays and phosphate concentrations presented in table 6.5. A S/N ratio of 0.84 mol/mol was maintained constant in all experiments.

Table 6.5 Assays to evaluate the effect of phosphate concentration

Assay	1	2	3	4	5
PO_4^{3-}-P (mg·L^{-1})	0	213	463	695	927

6.3.2 Methodology

The methodology for activities assays was previously described in chapter 2

6.3.3 Analytical methods

Analytical methods were previously described in Chapter 2.

6.4 RESULTS

6.4.1 Influence of sulphide and nitrate concentrations on autotrophic denitrification

6.4.1.1 Profiles of denitrification

The profiles of nitrogen production (Equation 6.1) utilizing different sulphide/nitrate concentrations are showed in Figure 6.1 and Table 6.6.

The main results show that, for assays performed at low concentration, denitrification immediately started after substrates addition, while a lag phase was detected for the other assays (Figure 6.1). The average time to reach maximum nitrogen production was between 70 to 250 minutes.

The percentages of denitrification were above to 80% in respect to the theoretical values (equation 6.1) for all assays with exception of assay 7 (300 mg $S^{2-} \cdot L^{-1}$) (Figure 6.2) where the maximum nitrate and sulphide concentration were tested. In this case the percentage of denitrification was very low (39.5%), nitrite and nitrous oxide being detected as products of denitrification, observing also a residual sulphide at end of assay (Table 6.6). Nitrite accumulation might be explained by the higher rate of nitrate reduction compared to that of nitrite reduction. This behaviour has been previously reported in assays performed under limiting thiosulphate conditions and stoichiometric condition with sulphide as donor electrons (Campos *et al*., 2008; Beristain *et al*., 2006). The presence of nitrous oxide suggests the inhibition of oxide reductase enzyme. It has been reported that high nitrite and sulphide concentrations provoked inhibition of enzymatic systems of denitrification mainly on nitrous oxide reductase enzyme (Sorensen *et al.,* 1980; Betlach and Tiedje 1981).

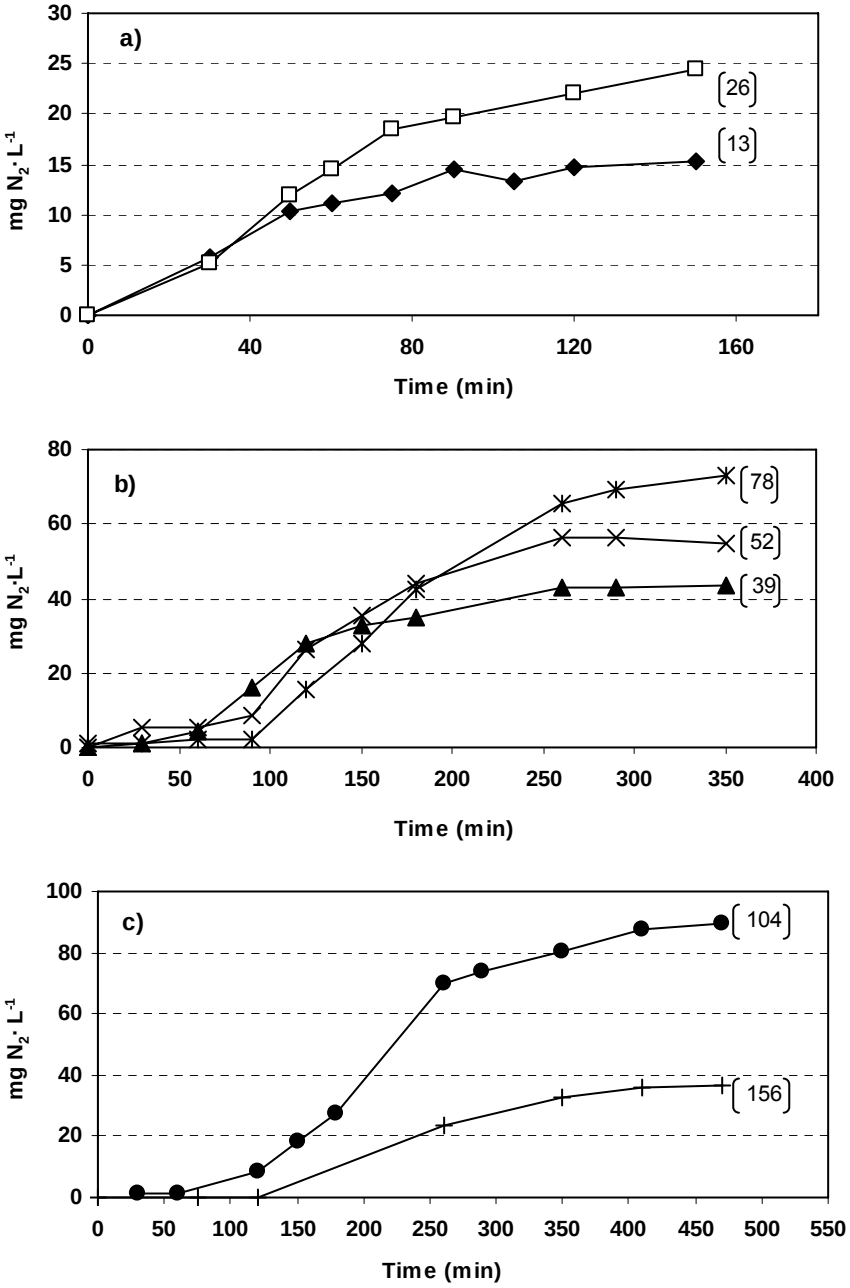

Figure 6.1 Profile of nitrogen production during assays at different sulphide and nitrate concentrations: assay (♦) 1, (□) 2, (▲) 3, (X) 4, (∗) 5, (●) 6 and (+) 7. Between brackets, theoretical N_2 production according to Eq. 6.1.

$$0.421\ H_2S + 0.421\ HS^- + NO_3^- + 0.346\ CO_2 + 0.086\ HCO_3^- + 0.086\ NH_4^+$$
$$\rightarrow 0.842\ SO_4^{2-} + 0.5\ N_2 + 0.086\ C_5H_7O_2N + 0.434\ H_2O + 0.262\ H^+ \qquad [6.1]$$

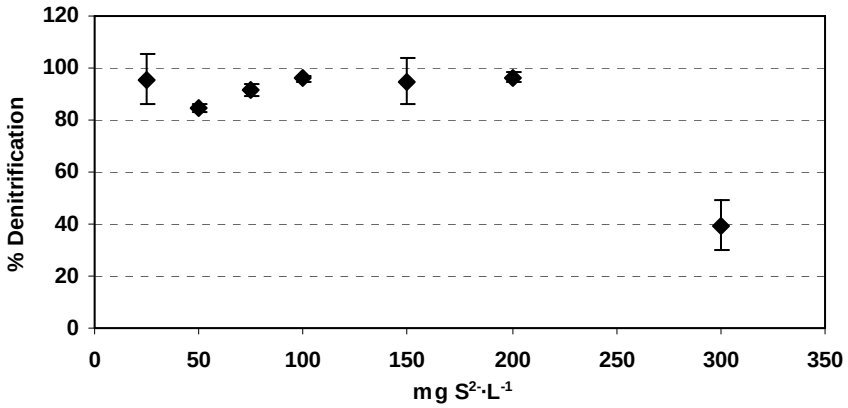

Figure 6.2 Influence of sulphide and nitrate concentrations on denitrification

It should be noted that, initial pH values were increased as nitrate and sulphide concentration increased (Table 6.6) reaching values close to 10 for assay 7 (300 mg $S^{2-}{\cdot}L^{-1}$), being in this case the denitrification strongly affected. This result agrees with those reported in literature for thiosulphate (Oh *et al*., 2000; Claus and Kutzner 1985).

However, during operation of the autotrophic denitrifying reactor (Chapter 4) when high values of pH were registered denitrification was not practically affected while the sulfoxidation was strongly affected.

Table 6.6 Influence of sulphide and nitrate concentrations on autotrophic denitrification

Assay	1	2	3	4	5	6	7
pH initial	7.19	7.34	7.42	7.58	7.68	7.91	8.88
pH final	7.33 ± 0.07	7.40 ± 0.07	7.48 ± 0.07	7.68 ± 0.07	8.17 ± 0.07	8.92 ± 0.07	9.94 ± 0.07
NO_3^--N initial	13	26	39	52	78	104	156
NO_3^--N final	4.7 ± 0.9	2.1 ± 0.98	1.9 ± 1.42	2.1 ± 0.54	3.9 ± 6.78	3.0 ± 1.92	16.0 ± 2.62
N_2 final	15.2 ± 3.12	24.3 ± 1.96	43.4 ± 1.5	54.5 ± 0.96	72.8 ± 4.11	89.8 ± 2.03	8.4 ± 1.78
NO_2^--N final	0.55 ± 0.21	1.88 ± 0.66	1.35 ± 0.78	0.0 ± 0.0	0.0 ± 0.0	0.65 ± 0.37	50.4 ± 2.3
N_2O-N final	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	27.8 ± 18.0
S^{2-} initial	25	50	75	100	150	200	300
S^{2-} final	2.81	0.0	0.0	6.0	0.0	2.64	17.4
% Denitrification	95.7 ± 9.6	84.6 ± 1.6	91.6 ± 2.4	96.0 ± 1.06	95.0 ± 8.7	91.5 ± 2.16	39.5 ± 9.8
Specific activity	63.6 ± 10.1	101.1 ± 24.7	109.8 ± 14.2	147.7 ± 14.6	158.5 ± 15.3	154.3 ± 5.01	55.0 ± 8.36

Units: concentrations (mg L^{-1}), specific activity (mg N_2·(g $VSS)^{-1}$·d^{-1})

6.4.1.2 Specific denitrifying activities

The specific denitrifying activities at different sulphide and nitrate concentrations (S/N 0.84 mol), are presented in Figure 6.3, with a maximum value of 158.5 mg $N_2 \cdot gVSS^{-1} \cdot d^{-1}$ for assay 5 (150 mg $S^{2-} \cdot L^{-1}$), this value being half than previously reported by Reyes-Avila *et al*. (2004) at stoichiometric ratio (104 mg $S^{2-} \cdot L^{-1}$).

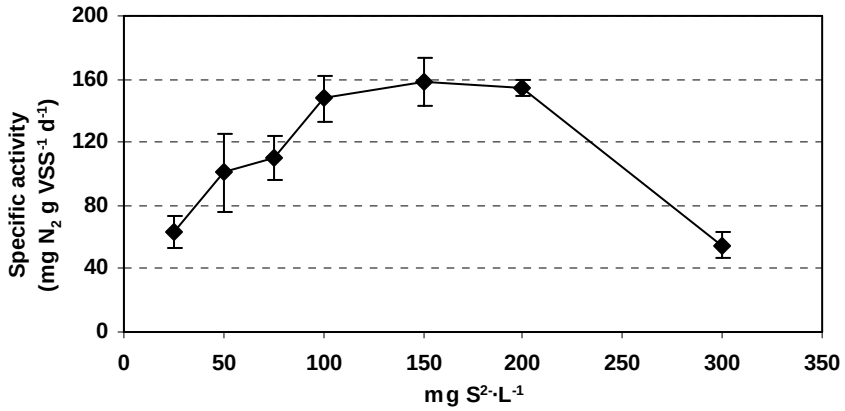

Figure 6.3 Specific denitrifying activities for different assays

In the assay 7, with 300 mg $S^{2-} \cdot L^{-1}$ the activity sharply decayed until 55.0 mg $N_2 \cdot gVSS^{-1} \cdot d^{-1}$. This fact could be due to the high sulphide concentration which increasing the initial pH close to 10.0, The loss of denitrification and specific denitrifying activity could be also associated to the presence of nitrite. It has been widely reported that nitrite has an inhibitory effect on denitrification even at low concentrations of 30-60 mg NO_2^--$N \cdot L^{-1}$ (Claus and Kutzner 1985; Krishnakumar and Manilal 1999). Campos *et al.* (2008) suggest that nitrite accumulation depends on S/N ratio used and initial nitrate concentration and that the control of both parameters is necessary to avoid the inhibitory effect of nitrite.

According to these results, the maximum sulphide loading rate under stoichiometric condition should not exceed of 200 mg $S^{2-} \cdot L^{-1}$. However, an autotrophic denitrifying reactor (Chapter 4) was efficiently operated at 450 mg $S^{2-} \cdot L^{-1}$ (sulphide limiting conditions) and the specific activity registered at the end of the operation was of 318 mg $S^{2-} \cdot g\ VSS^{-1} \cdot d^{-1}$, where only 27.5% of nitrate reduced was detected as nitrite (Table 4.3, chapter 4).

In this case the autotrophic denitrification was carried out in sequential batch reactors. One of the advantages of these systems is that they can easily be adapted for

continuous variation of pollutant concentrations (Mace and Mata-Alvarez, 2002), allowing also to avoid the accumulation of intermediates of denitrification, nitrous oxide and nitrite, when high nitrate and sulphide concentrations would be treated.

The batch assays here presented were carried out in closed vials, with a possible accumulation of denitrification intermediates (nitrite and nitrous oxide). Results of the assay with the maximum nitrate concentration tested show the presence of nitrite and nitrous oxide. The accumulation of these intermediates could be explained by the different reaction rates. An *et al*. (2010) reported values of sulphide and nitrate removal rates of 2.06 mM $S^{2-}\cdot h^{-1}$ and 1.05 mM $NO_3^-\cdot h^{-1}$ respectively and nitrite reduction rate of 0.05 mM $NO_2^-\cdot h^{-1}$. The high differences between nitrate and nitrite reduction rates agree with nitrite accumulation in this assay.

The low denitrification and specific activity values suggest an inhibition effect by substrate, in this work by nitrate concentration.

There are several studies reporting kinetic data for autotrophic denitrification, most of them carried out with thiosulphate as electron donor (Kim and Son. 2000; Oh *et al.* 2000; Claus and Kutzner 1985), and some others with elemental sulphur and sulphide (Zeng and Zhang, 2000; Gadekar *et al*. 2006). The main kinetics parameters reported are: μ_{max}, K_d, K_s and Y, however the kinetic parameter for inhibition by nitrate has not been reported.

The rate of substrate utilization can be diminished at different levels due to the presence of inhibitory compounds. In some cases, the inhibitor affects a single enzyme active in substrate utilization. In other cases the inhibitor can affect some more general cell functions, such as respiration processes. It can provoke indirect effects, such as the reduction of biomass levels slowing the utilization of a particular substrate, and the reduction of the flow of electrons (Ritman B.E. 2001).

In order to determined the inhibitory effect of nitrate concentration over autotrophic denitrification, the Haldane model was used to model the inhibition by nitrate. The Haldane model (equation 6.2) is one of the most commonly used to describe the self-inhibitory effect of a compound on its own transformation (Hao *et al.,* 2002; Sahinkaya and Dilek. 2005).

$$V = \frac{v_{\max} \cdot S}{Ks + S + \frac{S^2}{Ki}} \qquad [6.2]$$

Where:

V= production rate

v_{max}= maximum rate

S= substrate concentration

K*s*= Affinity constant

K*i*= Inhibition constant

Application of experimental data to the Haldane equation gave a good fit to the experimental data for this assay (Figure 6.4) as r^2 was observed to be 0.852. Another models were applied (Monod, Tessier and Mosser), giving lower values of r^2 than Haldane model.

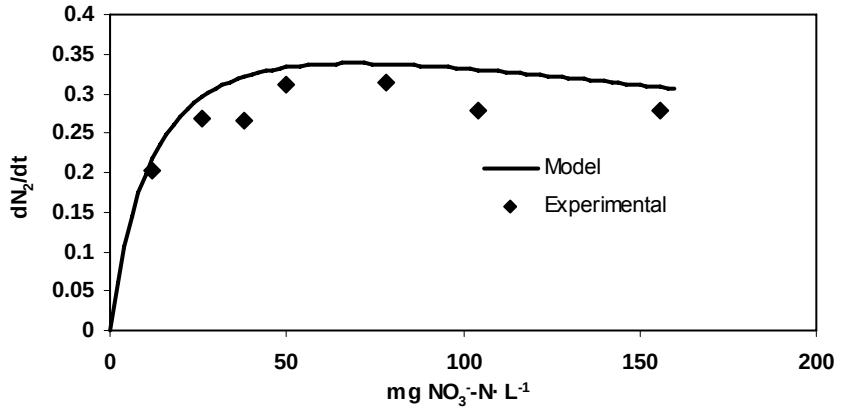

Figure 6.4 Experimental data (♦) and simulation used Haldane´s model (—)

A least-square error method with the help of MATLAB 7.10 (R2010a) was used to obtain the kinetic parameters. Haldane parameters were obtained as V_{max}= 0.470 ± 0.001 mg $N_2 \cdot L^{-1} \cdot min^{-1}$, K*s*= 13.51 ± 0.11 mg $NO_3^-\text{-}N \cdot L^{-1}$ and K*i*= 355 ± 4.4 mg L^{-1}. Therefore the experimental data can be expressed by the kinetic equation:

$$V = \frac{0.470 \cdot S}{13.51 + S + \frac{S^2}{355}} \qquad [6.3]$$

These kinetic parameters may be used as a tool for developing and designing an autotrophic denitrification process using sulphide as an electron donor.

The obtained value for Ks= 13.51 ± 0.11 mg NO_3^--N·L^{-1} was close to the ones reported in literature 3 – 10 mg NO_3^--N·L^{-1} when thiosulphate is used as an electron donor (Kim and Son. 2000; Oh *et al.,* 2000).

The value of inhibition coefficient obtained was K*i*= 355 ± 4.4 mg L^{-1}, suggesting that the maximum nitrate concentration applied at a system should not exceed this concentration. However, this value does not agree with the ones reported in literature, where the maximum nitrate concentration affecting the denitrification is between 450 and 2000 mg NO_3^--N·L^{-1} but when thiosulphate is used as electron donor (Claus and Kutzner 1985; Kim and Son. 2000; Oh *et al.,* 2000).

6.4.2 Influence of ammonium concentration on autotrophic denitrification

During this set of assays it was not practically detected any special effect of ammonium concentration on denitrification and specific denitrifying activity, obtaining denitrification efficiencies close to 100% for all assays and a practically constant specific denitrifying activity with an average value of 150 mg N_2·gVSS^{-1}·d^{-1} ± 6.8 (Figure 6.4, Table 6.7).

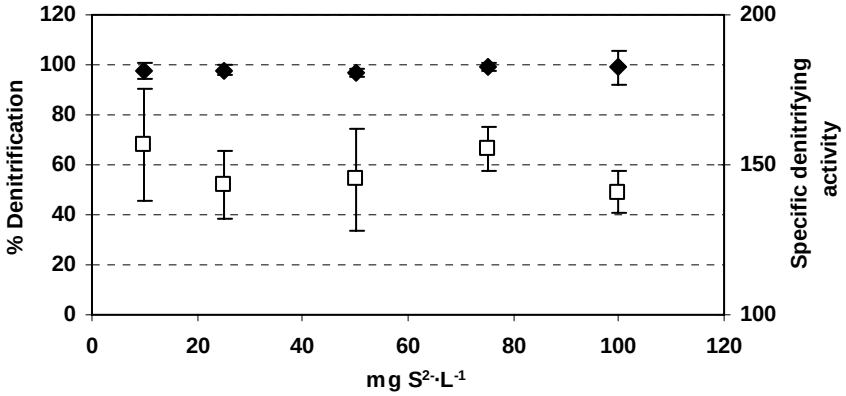

Figure 6.4 Influence of ammonium concentration on denitrification (♦) and specific denitrifying activity (□)

Table 6.7 Influence of ammonium concentrations on autotrophic denitrification

Assay	1	2	3	4	5
pH initial	7.78	7.73	7.75	7.77	7.78
pH final	7.88 ± 0.11	7.78 ± 0.04	7.7 ± 0.11	7.7 ± 0.05	7.7 ± 0.12
NO_3^--N intial	39	39	39	39	39
NO_3^--N final	1.1 ± 0.0	0.58 ± 0.02	1.2 ± 0.66	0.26 ± 0.05	0.32 ± 0.12
NH_4^+- N	10	25	50	75	100
N_2 final	35.5 ± 3.48	35.9 ± 1.80	38.8 ± 1.83	40.2 ± 1.54	32 ± 6.86
NO_2^--N final	4.9 ± 0.21	0.0	3.4 ± 2.4	2.76 ± 0.55	2.62± 0.03
S^{2-} initial	75	75	75	75	75
S^{2-} final	0.0	0.0	0.0	0.0	0.0
% denitrification	97.7 ± 3.8	98.0 ± 1.8	96.8 ± 1.8	99.3 ± 1.5	99.0 ± 6.5
Specific activity	156.8 ± 18.8	143.4 ± 11.1	145.1 ± 17.0	155.3 ± 7.2	141.0 ± 7.3

Units: concentrations (mg L^{-1}), specific activity (mg $N_2 \cdot g\ VSS^{-1} \cdot d^{-1}$)

6.4.3 Study of the influence of sulphide and nitrite concentrations on autotrophic denitrification

6.4.3.1 Profiles of denitrification

The profiles of nitrogen production at different sulphide/nitrite concentrations are showed in Figure 6.5 and Table 6.8.

During the assays performed at the lower concentrations (from 29 to 116 mg NO_2^--N L^{-1}) the denitrification started immediately after the addition of substrates, reaching the maximum value between 60 and 180 minutes (Figure 6.5a), while for assays performed from 174 to 348 mg NO_2^--N L^{-1} a lag phase of 90 minutes was registered before of denitrification started and finished in 300, 350 and 485 minutes respectively (Figure 6.5b).

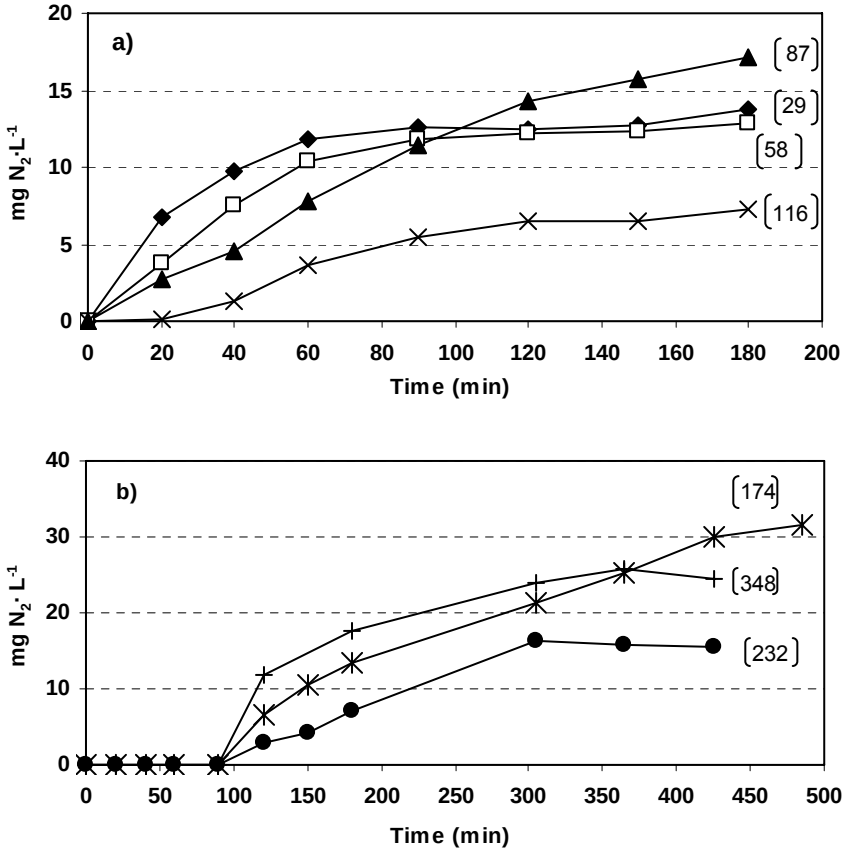

Figure 6.5 Profile of nitrogen production during assays at different sulphide and nitrite concentrations, assays: 1 (♦), 2 (□), 3 (▲), 4 (X), 5 (∗), 6 (●) and 7 (+). Between brackets, theoretical N_2 production according to Eq. 6.1.

In these experiments lower efficiencies of denitrification were registered, even at low nitrite and sulphide concentrations (Table 6.8) where the maximum percentage of denitrification was of 79%. This result agrees with the one previously reported by Claus and Kutzner (1985) and Oh *et al*. (2000) with thiosulphate as electron donor.

The percentage inhibition observed on denitrification is show in figure 6.6. The value of IC_{50} (concentration causing 50% inhibition) on the basis of denitrification was found to be 48 mg NO_2^--N L^{-1}. This value agrees with reported in literature, where the complete inhibition of autotrophic denitrification by nitrite is reported between 100 to 150 mg NO_2^--N L^{-1} (Claus and Kutzner 1985 and Oh *et al*., 2000). However, works performed by Mahmood *et al.* (2007) reported for enriched biomass with nitrite as electron acceptor, a percentage of nitrite reduction close to 90% at a nitrite concentration of 73 mg NO_2^--N L^{-1}.

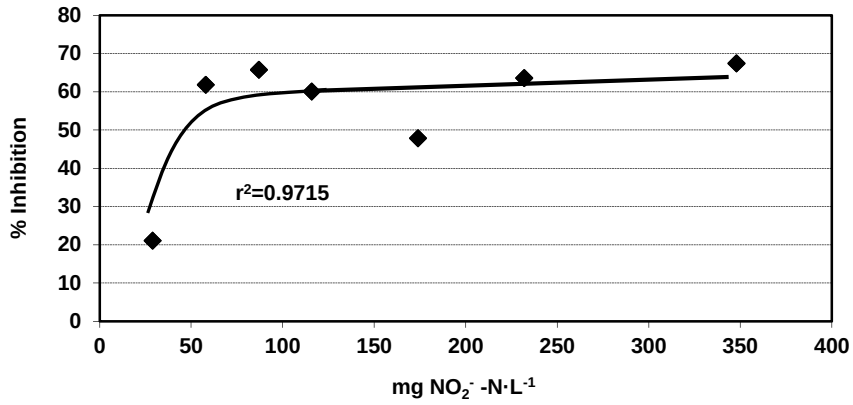

Figure 6.6 Percentage of inhibition of autotrophic denitrification caused by nitrite

During this assays nitrous oxide was detected as intermediate of denitrification, the amount of nitrous oxide produced being around 33% for most of assays with exception of assay 1 where this percentage was less than 5% (Figure 6.7). As previously mentioned the presence of nitrous oxide suggests inhibition of nitrous oxide reductase enzyme.

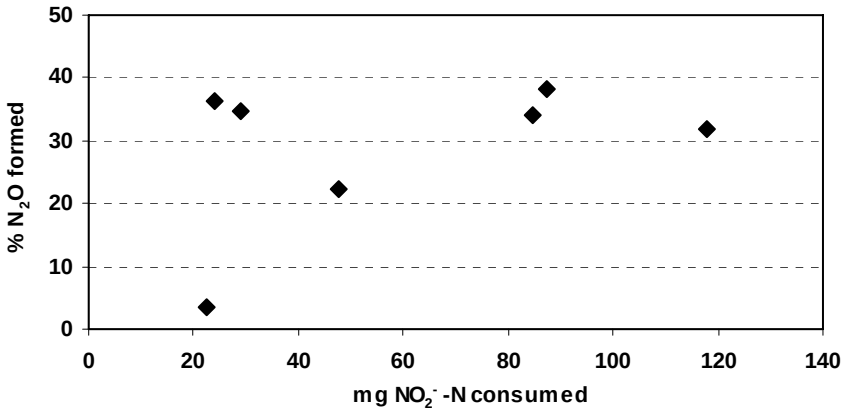

Figure 6.7 Percentage of nitrous oxide formation

Table 6.8 Influence of sulphide and nitrite concentrations on autotrophic denitrification

Assay	1	2	3	4	5	6	7
pH initial	7.71	7.86	7.95	8.09	8.36	8.77	9.35
pH final	7.73 ± 0.14	8.0 ± 0.14	8.2 ± 0.33	8.8 ± 0.61	9.41 ± 0.07	9.4 ± 0.35	10.1 ± 0.16
NO_2-N initial	29	58	87	116	174	232	348
NO_2-N final	6.5 ± 1.64	34 ± 8.5	58.1 ± 0.42	68.3 ± 3.1	89.3 ± 5.0	144.5 ± 43.0	230 ± 40.0
S^{2-} initial	25	50	75	100	150	200	300
S^{2-} final	0.0	1.04	1.2	1.3	3.16	8.5	63.3
N_2 final	20.5 ± 1.01	12.6± 1.87	15.0 ± 4.4	7.3 ± 2.45	13.6 ± 8.5	6.7 ± 4.2	9.2 ± 3.78
N_2O-N final	0.78 ± 1.86	8.7 ± 0.5	10.0 ± 4.8	10.6 ± 4.5	29.0 ± 6.8	33.3 ± 6.3	37.6 ± 6.5
% denitrification	79.0 ± 5.3	38.2 ± 15.4	34.3 ± 0.47	40.0 ± 2.7	52.2 ± 2.7	36.5 ± 18.8	32.6 ± 0.6
Specific activity	78.0 ± 11.2	68.4 ± 11.0	47.1 ± 7.4	32.1 ± 2.1	24.6 ± 12.2	25.1 ± 8.2	29.1 ± 5.8

Units: concentrations (mg L^{-1}), specific activity (mg $N_2 \cdot g\ VSS^{-1} \cdot d^{-1}$)

6.4.3.2 Specific denitrifying activities

The profile of specific denitrifying activities is shown in the Figure 6.8. The specific activity measured at the lowest concentration was 78 mg N_2 g·VSS^{-1}·d^{-1}, this value being similar to the one calculated for assay with nitrate (Table 6.6) at 13 mg NO_3-N·L^{-1} and 25 mg S^{2-}·L^{-1}. Although these assays were carried out under the same nitrate conditions, stoichiometric ratio, the specific activity for this experiment was strongly affected by nitrite concentrations which decrease until values of 30 mg N_2 g·VSS^{-1}·d^{-1} (Table 6.8). Inhibition of denitrification has been widely reported in the presence of nitrite even at low concentrations (Krishnakumar and Manilal 1999; Claus and Kutzner 1985).

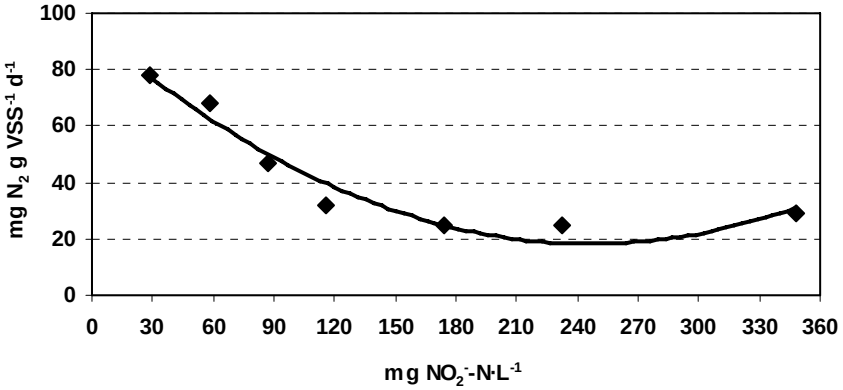

Figure 6.8 Specific activities with different sulphide/nitrite concentrations

The presence of nitrous oxide suggests inhibition of the final stage of denitrification where nitrous oxide is reducing to nitrogen gas by mean of nitrous oxide reductase. Several hypotheses have been proposed to explain this inhibition: **a)** Inhibition by sulphide: Sorensen *et al*. (1980) and Knowles (1982) postulate that sulphide provokes a strong inhibition of nitrous oxide reduction on heterotrophic denitrification. **b)** Inhibition or deactivation of nitrous oxide reductase in presence of oxygen has been widely reported even at very low oxygen concentrations (Wrage *et al.,* 2001; Körner and Zumft 1989; Berks *et al*., 1993). **c)** Nitrite accumulation may preferentially stimulate the synthesis of nitrite reductase enzyme, regarding nitrate reductase and nitrous oxide reductase enzymes presenting a decoupling of enzymatic system (Betlach and Tiedje 1981; Körner and Zumft 1989).

The inhibition of denitrification cannot be attributed to the sulphide concentration, due to the fact that when different sulphide/nitrate concentrations were tested (section 6.4.1), no inhibitory effect was detected on denitrification for concentrations from 25 to 200 mg $S^{2-} \cdot L^{-1}$. Only for the highest sulphide and nitrate concentrations the denitrification was strongly affected but not so the sulphide oxidation. However in the presence of nitrite as electron acceptor the denitrification was strongly inhibited even at low concentration.

Due to the fact that the assays were carried out under anoxic conditions the hypothesis of inhibition by oxygen does not explain the results obtained in this assay.

Therefore the inhibition of denitrification is attributed to high nitrite concentration and the presence of nitrous oxide. These results disagree with Mahmood *et al*. (2007) who reported efficiencies of denitrification of 90% in batch assays under nitrite limiting conditions and close to 80% under stoichiometric condition for a continuous reactor. These high efficiencies can be explained by the fact that they used sludge enriched with nitrite.

6.4.4 Influence of temperature on autotrophic denitrification

During this set of assays, it was observed that at temperatures of 30 and 35 ºC the nitrogen production started immediately after addition of substrates and the maximum production was reached in approximately 90 minutes (Figure 6.9), being the values of denitrification 83.6 and 97% respectively (Figure 6.10, Table 6.9). This behaviour disagrees with previously reported by Claus and Kutzner (1985) and Koenig and Liu (2004) who reported maximum denitrification at 30 ºC while at 35 ºC the denitrification declined between 10 to 20%, however it agrees with Oh *et al.* (2000) who observed that denitrification is higher at 35 ºC than at 30 ºC.

During the assay performed at 25 ºC a lag phase of 50 minutes was registered and a loss of 40% of denitrification was detected (Figures 6.9 and 6.10). This behaviour agrees with previously reported using thiosulphate as electron donor (Oh *et al*., 2000; Koenig and Liu 2004).

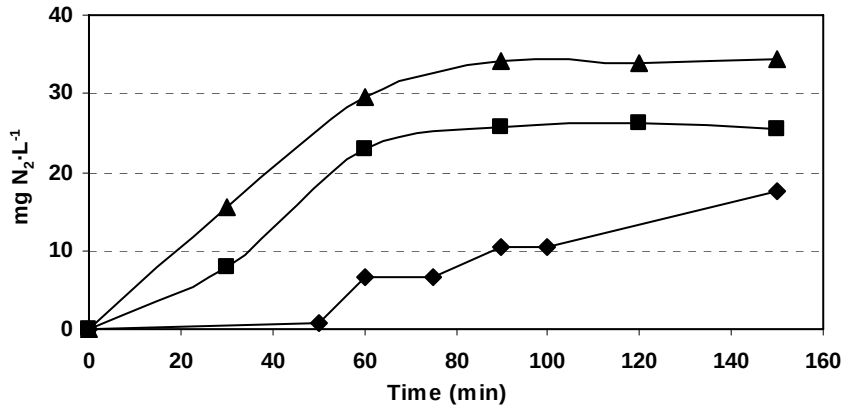

Figure 6.9 Assays performed at different temperatures: (♦) 25 º C, (■) 30 º C and (▲) 35 ºC.

Table 6.9 Influence of temperature on autotrophic denitrification

Assay	1	2	3
Temperature	25	30	35
pH initial	7.45	7.45	7.45
pH final	7.59 ± 0.03	7.56 ± 0.02	7.62 ± 0.06
NO_3-N initial	39	39	39
NO_3-N final	3.7 ± 4.8	8.4 ± 4.8	3.68 ± 0.53
N_2 final	20.8 ± 2.02	25.6± 0.81	34.3 ± 1.4
NO_2-N final	4.7 ± 2.9	2.7 ± 0.3	1.87 ± 0.46
S^{2-} initial	75	75	75
S^{2-} final	0.0	0.0	0.0
% denitrification	59.0 ± 2.04	83.6 ± 0.41	97.0 ± 1.41
Specific activity	48.3 ± 14.0	149.0 ± 11.0	188.0 ± 14.3

Units: concentrations (mg L^{-1}), specific activity (mg $N_2 \cdot g\ VSS^{-1} \cdot d^{-1}$), temperature (º C)

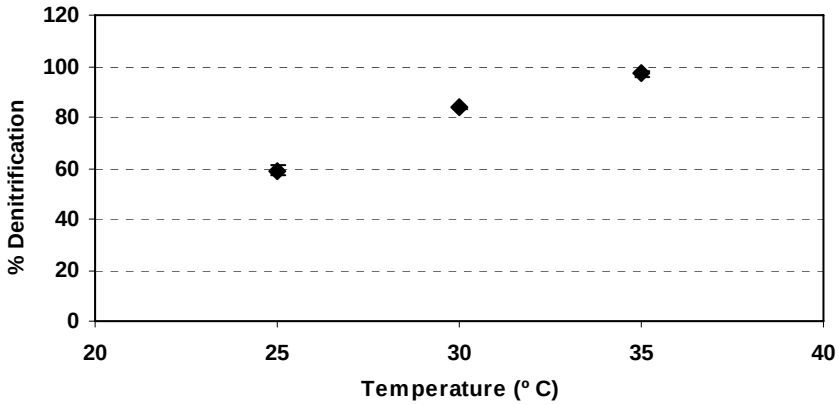

Figure 6.10 Influence of temperature on autotrophic denitrification

The specific denitrifying activity measured increased from 48.3 mg $N_2 \cdot gVSS^{-1} \cdot d^{-1}$ at 25 ºC to 188.0 mg $N_2 \cdot gVSS^{-1} \cdot d^{-1}$ at 35 ºC (Figure 6.11).Similar results are reported by Campos *et al*. (2008) using thiosulphate as electron donor.

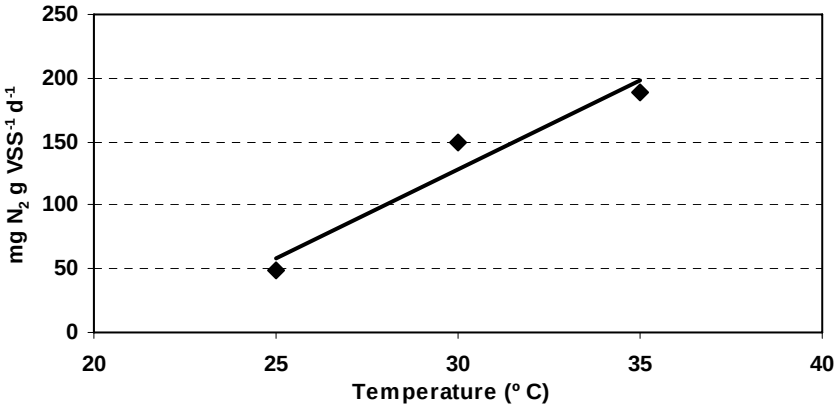

Figure 6.11 Influence of temperature on specific activities

6.4.5 Influence of cross-effect of temperature/pH/NH_3 on autotrophic denitrification

6.4.5.1 Profiles of denitrification

The individual effect of temperature was similar to the previously observed in experiments 6.4.4 presenting lag phases at low temperature (15 and 25 ºC) while at 35 ºC the denitrification starts immediately after addition of substrates (Figure 6.12).

Denitrification was strongly influenced by temperature. It was observed that below 30 º C, which is the optimum reported for autotrophic denitrification (Claus and Kutzner 1985) the denitrification declines until 58.7% and 36% for the assays performed at 25 ºC and 15 ºC respectively (Table 6.10).

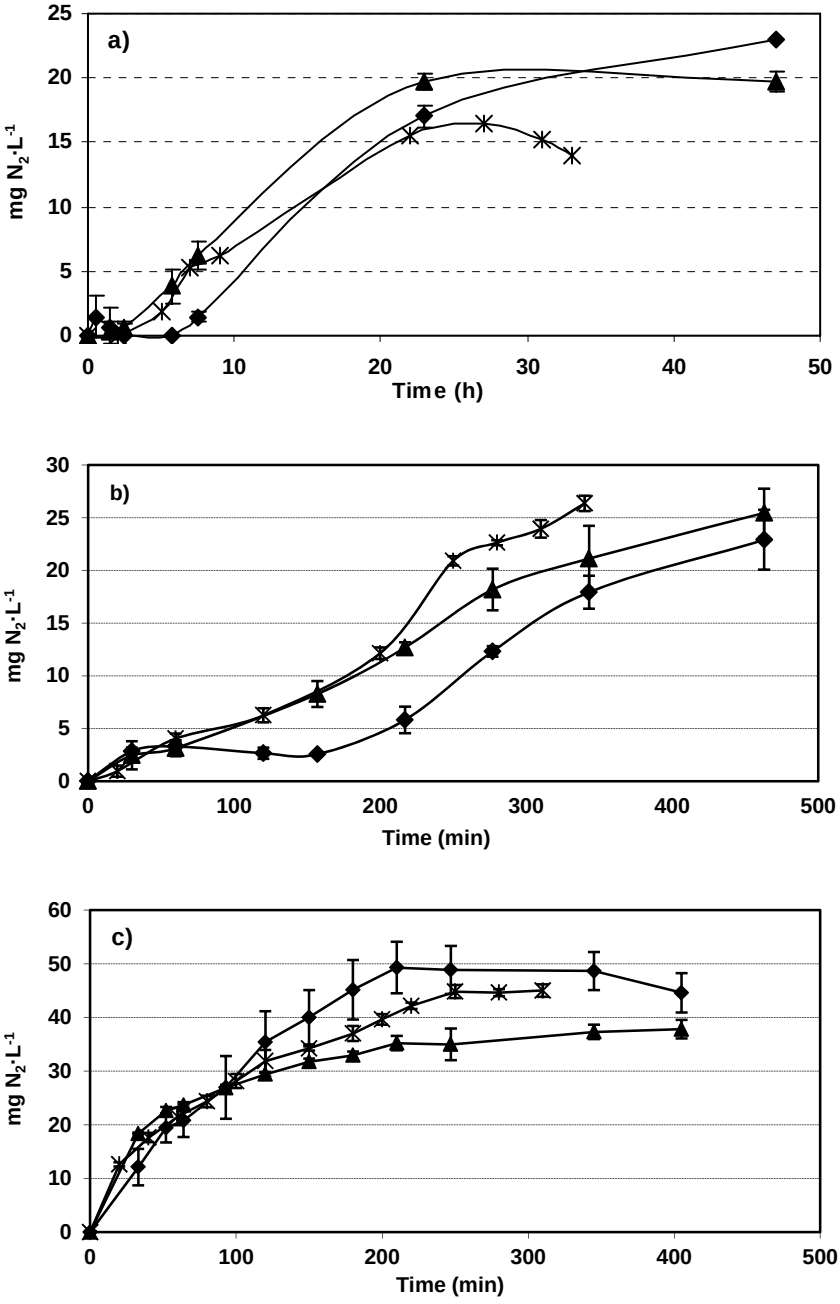

Figure 6.12 Influence of Temperature and pH on autotrophic denitrification: a) 15 ºC, b) 25 ºC and c) 35 ºC (♦) pH 7.0, (▲) pH 8 and (∗) pH 9 (39 mgNO_3^--N·L^{-1} for all assays).

Table 6.10 Influence of cross-effect of temperature/pH/NH_3 on autotrophic denitrification

Assay	1	2	3	4	5	6	7	8	9
Temperature		15 ° C			25 ºC			35 ºC	
pH initial	7.11	8.08	9.05	7.11	8.08	9.05	7.11	8.08	9.05
pH final	7.3 ± 0.01	8.33 ± 0.0		7.23 ± 0.01	8.36 ± 0.03	9.07 ± 0.01	7.23 ± 0.01	8.43 ± 0.0	9.07 ± 0.01
NH_3-N initial	0.27	2.67	21.3	0.56	5.37	36.2	1.12	10.1	53.1
NO_3-N initial	39	39	39	39	39	39	39	39	39
NO_3^--N final	0.0	0.0		0.0	0.0	12.2 ± 10.1	0.0	0.0	8.8 ± 6.3
N_2 final	23.0 ± 1.5	19.7 ± 0.82	16.4 ± 0.97	22.9 ± 2.8	21.1 ± 3.1	26.3 ± 0.72	44.6 ± 3.6	37.8 ± 1.7	45.0 ± 1.14
S^{2-} initial	75	75	75	75	75	75	75	75	75
S^{2-} final	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0
% denitrification	58.9 ± 3.9	50.5 ± 2.1	42.0 ± 2.2	58.7 ± 7.3	65.3 ± 5.8	91.7 ± 6.5	100 ± 9.4	96.7 ± 4.5	100 ± 10.6
Specific activity	5.03 ± 0.3	4.67 ± 0.2	3.4 ± 0.16	27.0 ± 0.62	19.2 ± 1.8	30.6 ± 0.3	83.0 ± 10.3	145.0 ± 14.0	68.0 ± 4.5

Units: concentrations (mg L^{-1}), specific activity (mg N_2·g VSS^{-1}·d^{-1})

The optimum pH for autotrophic denitrification is reported between 7 to 8 (Oh *et al*. 2000; Claus and Kutzner 1985). In these assays the effect of pH on autotrophic denitrification was closely related to temperature; at 35 ºC the influence of pH on denitrification was practically negligible even at pH 9 (Figure 6.13, Table 6.10). This fact disagrees with results of Moon *et al*. (2004) and Oh *et al*. (2000) who reported complete inhibition of denitrification at pH 9, however Mahmood *et al*. (2008) reported complete denitrification at wide range of pH (5-11) but incomplete oxidation of sulphide to sulphate. Similar results were obtained during the operation of autotrophic reactor (Chapter 4) where at values of pH close to 10 in the effluent, sulphide was partially oxidized to elemental sulphur (Figure 4.2).

For assays performed at 25 ºC, at pH 7 and 8 the denitrification was affected decreasing until 58.7 and 65.3 % respectively; however at pH 9 high efficiency of denitrification was registered 91.7% (Table 6.10). These results agree with those reported by Mahmood *et al*. (2008).

The denitrification was affected at 15 ºC for all pH tested with a maximum value at pH 7 (58.9%) while at pH 9 the denitrification was of 42%.

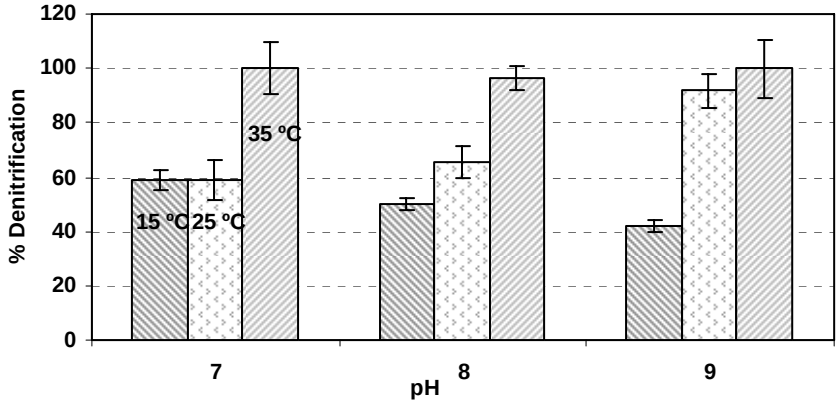

Figure 6.13 Influence of temperature and pH on denitrification

6.4.5.2 Specific denitrifying activities

As denitrification also specific activities were strongly influenced by temperature, being obtaining a maximum value at 35 ºC and pH 8 (145 mg N_2·g VSS^{-1}·d^{-1}) (Figure 6.14, Table 6.10), while at pH 7 and 9 the values obtained were 83 and 68 mg N_2·g VSS^{-1}·d^{-1} respectively. However when the assay was performed at 25 ºC the specific activities

sharply decayed until values of 27, 19 and 30 mg N_2·g VSS^{-1}·d^{-1} for pH 7, 8 and 9 respectively, while at 15 º C the activities were below 5.0 mg N_2·g VSS^{-1}·d^{-1} (Figure 6.14).

The values obtained in this assay at 25 and 35 ºC were slightly lower than that obtained during assay 6.4.4 at same temperature and pH 7.45, suggesting that the activity was strongly influenced by pH tested. This result could explained the lost of sulfo-oxidant activity observed during operation of autotrophic reactor (Chapter 4) where the activity decreased at pH values slightly alkaline.

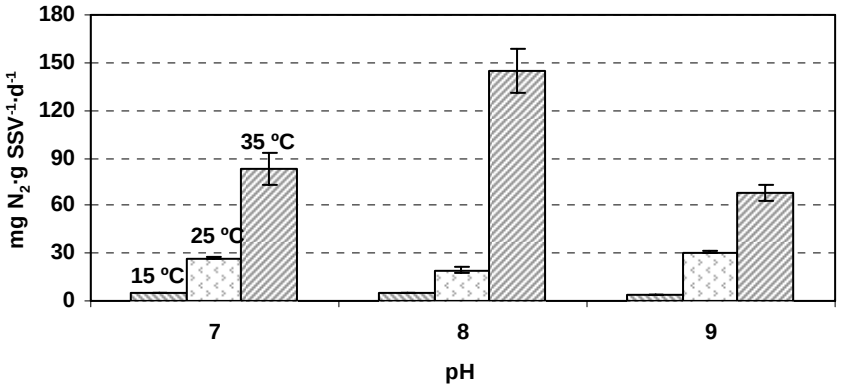

Figure 6.14 Influence of temperature and pH on specific activity

It is widely reported the inhibition of nitrification by free ammonia NH_3-N (0.1 – 1.0 mg NH_3-N L^{-1}) and free nitrous acid HNO_2-N (2.0 mgHNO_2-N L^{-1}) (Anthonisen *et al.* 1976; Van Hulle *et al*. 2007). However the results of this work do not show a correlation between free ammonium concentration and efficiency of denitrification and specific activities (Table 6.10). This behaviour agree with previously reported by Fernández *et al.* (2006) who reported no inhibitory effect on the heterotrophic denitrifying activity at concentrations up to 550 mg NH_3-N·L^{-1}.

As can be observed during the operation of autotrophic denitrifying reactor (Chapter 4) where high values of pH were registered, it can be concluded that the free ammonium formed, does not affected the denitrification.

6.4.6 Influence of phosphate concentration on the autotrophic denitrification

6.4.6.1 Profiles of denitrification

Figure 6.15 shows the influence of phosphate on autotrophic denitrification. It can be observed that the denitrification values fluctuate between 91 to 100% for assays performed

with different phosphate concentrations while in absence of phosphate denitrification declined until 86% (Table 6.11).

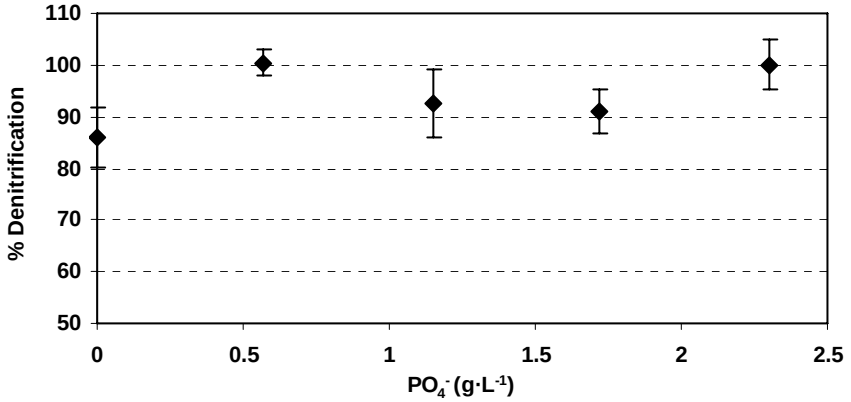

Figure 6.15 Influence of phosphate concentration on autotrophic denitrification

Table 6.11 Average performance of assays with different phosphate concentrations (S/N 0.84 mol)

Assay	1	2	3	4	5
pH initial	7.4	6.76	6.85	6.84	6.8
pH final	9.15 ± 0.03	7.34 ± 0.0	7.3 ± 0.01	7.14 ± 0.01	7.04 ± 0.02
PO_4^-	0.0	570	1150	1720	2300
NO_3^--N initial	39	39	39	39	39
NO_3^--N final	2.6 ± 0.7	4.9 ± 0.09	6.4 ± 4.7	1.5 ± 1.3	3.15 ± 1.0
N_2 final	31.3 ± 2.01	34.2± 0.9	32.9 ± 1.0	33.5 ± 1.6	36.8 ± 1.2
S^{2-} initial	75	75	75	75	75
S^{2-} final	0.0	0.0	0.0	0.0	0.0
% Denitrification	86 ± 5.6	100 ± 2.5	92.6 ± 6.6	91.1 ± 4.32	100 ± 4.78
Specific activity	35.8 ± 4.0	56.0 ± 3.3	50.8 ± 6.0	62.7 ± 3.4	66.1 ± 10.8

Units: concentrations (mg L^{-1}), specific activity (mg N_2·g VSS^{-1}·d^{-1})

6.4.6.2 Influence of phosphate concentration on the specific activity

While the influence of phosphate concentration on the autotrophic denitrification was low, this parameter affected the specific denitrifying activities which decline from 66 mg N_2·g VSS^{-1}·d^{-1} for higher phosphate concentration to 35 mg N_2·g VSS^{-1}·d^{-1} for assay free of phosphate (Figure 6.16). In literature has been reported that denitrification is affected by a lack of source of phosphate, probably due to the buffering capacity (Hunter 2003; Nugroho *et al.* 2002; Koenig and Liu 2004; Moon *et al.* 2008). This fact confirmed during operation of autotrophic denitrifying reactor (Chapter 4, Figure 4.2) where during first 63 days of

operation values of pH was gradually increased from 7.8 to 9.3 due to a low buffering capacity of the system also affecting to the specific sulphide activity (Figure 4.10), while once the phosphate concentration in the mineral medium was increased, it was possible to operate at high sulphide loading rates and the specific sulphide activity of the system was recovered. Because of the limited knowledge about the role of phosphate on autotrophic denitrification more studies are need to understand this relationship.

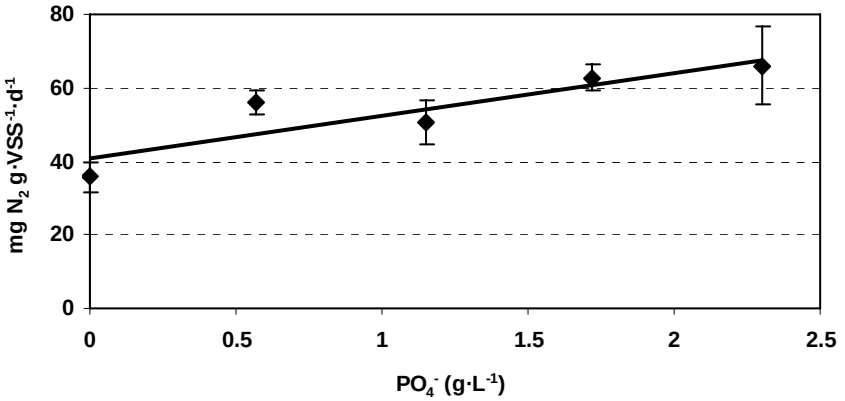

Figure 6.16 Influence of phosphate concentrations on specific denitrifying activity

6.5 CONCLUSIONS

The results obtained at different sulphide/nitrate concentrations could be explained as inhibition by substrate, the Haldane´s model seems to be an adequate expression as nitrate inhibits its own reduction to nitrogen gas at high concentrations. The following parameters were determined: V_{max}= 0.470 ± 0.0013 mg $N_2 \cdot L^{-1} \cdot min^{-1}$, Ks= 13.51 ± 0.113 mg L^{-1} and K*i*=355 ± 4.38 mg L^{-1} with an r^2 of 0.852.

Ammonium concentration did not present negative effect both on denitrification and specific activity under stoichiometric conditions of nitrate to sulphide.

Inhibition of autotrophic denitrification by nitrite was confirmed and a value of IC_{50} of 48 mg NO_2^--N·L^{-1} was obtained. The presence of high concentration of nitrous oxide suggests that the inhibition was at level of nitrous oxide reductase.

Complete denitrification and maximum specific activity were registered at temperature of 35 ºC and pH of 8.0.

The phosphate does not provoke any inhibitory effect on denitrification however the specific activity being slightly affected in the absence of phosphates.

Nitrite, nitrous oxide concentration and temperature are the main parameters that affected of autotrophic denitrification.

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Chapter 7.

Population Dynamics in Autotrophic Denitrifying Reactors

Summary

In this chapter the microbial communities of Autotrophic Denitrifying and Nitrifying reactors were analyzed by means of molecular techniques (DGEE, PCR and FISH). Biomass samples were collected from three autotrophic denitrifying reactors and one nitrifying reactor; i) a batch reactor for the enrichment of autotrophic denitrifying biomass, ii) a mature autotrophic denitrifying reactor fed with mineral medium, iii) a mature autotrophic denitrifying reactor fed with the effluent from a nitrifying reactor and, iv) a nitrifying reactor fed with the effluent from an anaerobic digester treating the wastewater from a fish canning industry. Analysis performed with the FISH technique revealed that the microbial communities present in the enrichment and mature autotrophic denitrifying reactors were characterized by the presence of *Thiobacillus denitrificans.* This population was influenced by changes in the pH value. However no negative effect was observed when the feeding media was switched by the effluent from the nitrifying reactor. The phylogenetic study of the bacterial community showed that the predominant population was close to *Thiobacillus denitrificans* and *Bacteroidetes* with 99% similarity. Another two microorganisms able to use sulphur compounds were also detected, *Chlorobi* and candidate division JS1 with 95% and 94% similarity, respectively. Furthermore *Stenotrophomonas* was identified with 97% similarity and some members of this genus are known to be able to use nitrate under semi-anaerobic conditions.

7.1 INTRODUCTION

Knowledge of the microbial ecology in biological wastewater treatment systems allows for a better understanding of the processes involved, the factors that regulate their activities and the best operational parameters (Koenig *et al*., 2005; Figueroa *et al*., 2008; Fernández *et al*., 2008).

Simultaneous removal of reduced sulphur compounds and nitrate or nitrite from drinking water, ground water, and industrial wastewaters has been widely accomplished by means of autotrophic denitrifying processes (Soares 2002; Moon *et al*., 2004; Vaioupolou *et al*., 2005). However information about the microbial communities involved in such processes is scarce. Little is known about the microbial diversity of autotrophic denitrifying micro-organisms and only two species, *Thiobacillus denitrificans* and *Sulfurimonas denitrificans* (before known as *Thiomicrospira denitrificans,* Takai *et al., 2006*), have been identified as able to carry out the denitrification in the presence of sulphur reduced compounds acting as electron donor (Claus and Kutzner 1985; Oh *et al*., 2000; Gadekar *et al*., 2006). Facultative bacteria, able to grow autotrophically or heterotrophically, as *Thiobacillus versutus* and *Thiobacillus thyasiris* have been reported to exhibit nitrate reduction coupled to sulphide reduction (Chazal and Lens, 2000). Furthermore *Paracoccus denitrificans* has been found to be able to use hydrogen as well as sulphur reduced compounds as electron donor (Mateju *et al*., 1992).

Nowadays molecular techniques in combination with classical methodologies are recognised as a very useful tool for the identification of micro-organisms, the evaluation of population dynamics and the morphological and biochemical characterization of the biomass involved in biological process in treatment systems (Nielsen *et al.,* 2009; Koenig *et al*., 2005; Sinyambalapitiya and Blackall 2005; Byun *et al.,* 2008).

Many molecular studies have been performed in order to characterize the distribution of autotrophic denitrifying populations mainly in sediments (Llobet-Brosa *et al*., 2002; Shao *et al.,* 2009; Bettar *et al*., 2006; Zhang *et al*., 2009). However, there is little information regarding the microbial ecology of autotrophic denitrifying reactors. Molecular techniques applied to the study of autotrophic denitrifying processes revealed that microbial diversity is heterogeneous and is influenced by the type of used inoculum and the characteristic of the wastewater. From the application of these techniques it has been observed that

Thiobacillus denitrificans is always detected no matter which type of wastewater is used (Koenig *et al*., 2005; Fernández *et al.,* 2008; Byun *et al*., 2008; Park *et al*., 2008).

7.2 OBJECTIVE

Nowadays autotrophic denitrification technologies are widely known and the operational parameters are well established; however, little is known about the microbial diversity of the biomass performing this process. For this reason the aim of the work presented in this chapter is:

To characterize by means of molecular techniques the evolution experienced by the microbial community of the inoculum (anaerobic sludge) during its enrichment in autotrophic denitrifying micro-organisms and through the operation of the autotrophic denitrifying process in a mature culture fed with synthetic and industrial effluent. A similar study was performed with the biomass present in a nitrifying reactor producing the wastewater to feed the denitrifying autotrophic reactor.

7.3 MATERIALS AND METHODS

7.3.1 Reactors

Samples of four different reactors were collected. The performance of each reactor is summarized below:

i) Enrichment batch reactor (ER)

A batch reactor with a total volume of 5 L and a working volume of 1.5 L was operated during 100 days (detailed information about the operation of this reactor is provided in Chapter 3). Dimensions of the reactor were: height 0.6 m and inner diameter of 0.12 m. A thermostated bath was installed to control the temperature at 30 ± 1.0 ºC, and mixing was achieved with a mechanical stirrer at 150 rpm. The pH value was not controlled and ranged from 7.0 to 7.5. The reactor was inoculated with anaerobic sludge collected from a wastewater treatment plant treating the effluent of a fish canning industry. The

biomass concentration inside the system at the beginning of this study was of 19.7 g VSS L^{-1}. The reactor was operated in batch mode during one year fed with a mineral medium containing thiosulphate and nitrate at concentrations of 1.54 g $S_2O_3^{2-}$- S·L^{-1} and 0.247 g NO_3^- -N L^{-1} (Chapter 3).

ii) Autotrophic denitrifying reactor fed with sulphide as electron donor (M1)

The process was carried out in a SBR with a working volume of 1 L operated during 220 days (detailed information about the operation of this reactor is provided in Chapter 4). The reactor was inoculated with the enriched biomass from the previous batch reactor (Chapter 3). The temperature of operation was maintained at 30 ± 1 ºC by means of a thermostatic jacket. Complete mixture inside the reactor was achieved with a mechanical stirrer operated at 150 rpm. The hydraulic retention time (HRT) was fixed in 1 day. The reactor was flushed with a mixture of 95% Ar and 5% CO_2 to maintain anoxic conditions. This reactor operated at sulphide loading rates (SLR) from 200 mg S^{2-}·L^{-1}·d^{-1} to 450 mg S^{2-}·L^{-1}·d^{-1} while the nitrogen loading rates (NLR) were kept constant at 450 mg NO_3^--N·L^{-1}·d^{-1}. On stage III of operation values of pH close to 10 were registered. Under these conditions the sulphate production and the specific activity decreased which indicated that the autotrophic denitrifying population was affected by pH. Once the pH value was controlled, the oxidation of sulphide and the specific activities were restored.

iii) Autotrophic denitrifying reactor fed with the effluent from a nitrifying reactor and sulphide as electron donor (M2)

Autotrophic denitrification was carried out in a SBR operated at the same operational conditions (TRH, rpm, T) as the previous one (detailed information about the operation of this reactor is provided in Chapter 5). The biomass concentration inside the reactor at the beginning of the experiment was of 4.2 g VSS·L^{-1}. The reactor was fed with mineral medium for 121 days at a SLR of 142 mg S^{2-} L^{-1} d^{-1} and a NLR of 466 mg N·L^{-1}·d^{-1}. From day 122 on the nitrate source was supplied as the effluent from a nitrifying reactor. The reactor was operated at different nitrogen and sulphur loading rates, from 149 to 285 mg N·L^{-1}·d^{-1} and 156 to 203 mg S^{2-}·L^{-1}·d^{-1}, respectively. When the feeding was changed by the

nitrifying effluent the autotrophic denitrifying activity decreased, which indicated that this parameter exerted a certain negative effect on the autotrophic denitrifying population.

iv) Nitrifying reactor (NR)

An activated sludge unit with a working volume of 0.5 L coupled to an external settler of 1.0 L was used (detailed information about the operation of this reactor is provided in Chapter 5). The system was operated at a hydraulic retention time (HRT) of 1 day and at room temperature (25 ± 0.5 ºC), DO higher than 2 mg O_2 L^{-1} and the pH value from 7.5 to 8.5. The system was inoculated with activated sludge from a municipal wastewater treatment plant. The concentration of biomass inside the reactor at the beginning of the experiment was 1.52 g VSS·L^{-1}.The reactor was fed with the effluent from an anaerobic digester treating the wastewater from a fish canning industry.

7.3.2 Analytical methods

The pH value and the volatile suspended solids (VSS) concentration were determined according to the Standard Methods (APHA, 2005). Nitrite, nitrate and sulphate concentrations were determined by ion chromatography while sulphide and dissolved oxygen ones were measured by means of ion-selective electrodes (see Chapter 2 for further information).

7.3.3 DNA extraction

Source of biomass samples

DNA extraction was performed of samples collected from the inoculum of the enrichment reactor (ER) (sample A), from the inoculum of the autotrophic denitrifying reactor (M1) (sample B), on day 67 of operation (sample C) and two months after pH shock (sample D) and from the reactor fed with the nitrifying effluent (M2) (sample E) (Table 7.1).

Total community DNA was extracted from the harvested biomass following a bead beating protocol using a PowerSoil DNA soil extraction kit (MoBio Laboratories, Inc., Solano Beach, CA) according to the manufacturer´s instructions.

7.3.4 PCR- DGGE

Genomic DNAs were subjected to DGGE analysis as previously described (Alonso-Gutierrez *et. al*., 2009a). The 16S rRNA gene hypervariable regions of bacteria V3-V5 were amplified using primers 16F341-GC (5´-CCTACGGGAGGCAGCAG-3´) and 16R907 (5´-CCGTCAATTCCTTTRAGTTT-3´) (Yu and Morrison, 2004). Primer F341-GC included a GC clamp at the 5´end (5´-CGC CCG CCG CGCC CCG CGC CCG TCC CGC CGC CCC CGC CCG-3´).

PCRs were performed using Veriti Thermocycler (PE Applied Biosystems, Foster City, California, USA) in a volume of 50 µL containing 1.25 U of Taq (TaKaRa ExTaq Hot Start Version; TaKaRa Bio Inc., Otsu, Siga, Japan), 1x ExTaq Buffer (2 mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 0.5 µM of primers and 100 ng of template DNA. After 9 min of initial denaturation at 95 °C, a touchdown thermal profile protocol was carried out, and the annealing temperature was decreased by 1 °C per cycle from 65 °C to 55 °C; followed by 25 additional cycles of 1 min of denaturation at 94 °C, 1 min of primer annealing at 55 ºC, and 1.5 min of primer extension at 72 °C, followed by 10 min of final primer extension at 72 ºC.

Approximately 800 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel that was 0.75 mm thick with denaturing gradients and ranged from 40 to 75% denaturant concentrations (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1×TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA; pH 8.4) using an INGENY PhorU system (Ingeny, Goes, The Netherlands) at 100 V and 60 °C for 17 h. DGGE gels were stained with 1 x TAE buffer containing SybrGold (Molecular Probes, Inc., Eugene, OR, USA). Predominant DGGE bands were excised with a sterile razor blade, suspended in 50 µL sterilized MilliQ water, stored at 4 °C overnight, reamplified by PCR using primers F341-R907 and sequenced as described below.

7.3.5 Sequencing

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

Sequencing was accomplished using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) and an ABI PRISM 3700 automated sequencer (PE Applied Biosystems, Foster City, California, USA) following the manufacturer´s instructions.

7.3.6 Phylogenetic analysis

Phylogenetic analyses were performed as previously described by Alonso-Gutierrez *et al*. (2009b) with some modifications. Sequences were inspected, corrected and assembled into a single consensus sequence for each phylotype. After that, the sequences were examined with the BLAST search alignment tool comparison software (BLASTN) (Altschul *et al*., 1990) to detect the closest prokaryotic group to each sequence among the GenBank database. Sequences from all phylotypes were aligned with reference sequences obtained from GenBank using MAFFT (Katoh *et al*., 2005). The alignment obtained was transferred to MacClade (Maddison & Maddison, 2003) and finally edited using the MacClade program. The edited alignment was directly transferred to jMODELTEST software version 3.6 (Posada & Crandall, 1998) as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment. The jMODELTEST examines ML models, ranging from simple to complex. It was allowed for rate variation across sites, assuming a gamma distribution (0.4890) and a proportion of invariable sites (0.2580) estimated by using jMODELTEST (Akaike information criterion; Posada & Crandall, 1998Go). Base frequencies determined by using jMODELTEST for A, C, G and T were of 0.2759, 0.1844, 0.2797 and 0.2600, respectively, with the rate matrix of the substitution model being 1.0000 (AC), 1.8124 (AG), 1.0000 (AT), 1.0000 (CG), 2.9845 (CT) and 1.0000 (GT). We assessed the relative stability of the tree topology by using

1000 distance bootstrap replicates and 100 maximum-likelihood bootstrap replicates. The settings for bootstrap calculations were the same as those given above. These best-fit models of nucleotide evolution, calculated by jMODELTEST were incorporated into software PHYML (Guidon & Gascuel, 2003), which uses a single, fast, and accurate algorithm to estimate large phylogenies by ML. Finally, the trees created by PHYML were edited using the FIGTREE v1.1.2 software (http://tree.bio.ed.ac.uk/software/figtree/).

7.3.7 FLUORESCENT *in situ* HYBRIDIZATION (FISH)

The FISH technique was applied for the identification of the bacterial populations from the same samples used for the phylogenetic analysis (Table 7.1)

Biomass samples from the reactors 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 7.2. The used probes for *in situ* hybridization were 5´ labelled with the fluorochromes FITC and Cy3. Fluorescence signals were recorded with an acquisition system (Coolsnap, Roper Sicientific Photometrics) coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany).

The FISH probes used in this work were selected based on previous studies about autotrophic denitrifying reactors and the DGGE results from the present study (Table 7.2).

Table 7.1 Samples analyzed by FISH and DGGE techniques.

Reactor	Sample (day)	Specific activity (mg S·(g VSS)$^{-1}$·d^{-1})	FISH analysis	DGGE analysis
Enrichment Batch Reactor (ER) ($S_2O_3^{2-}/NO_3^-$)	0 (inoculum)	9.5[a]	√	A
	60	ND	√	NA
	180	ND	√	NA
Autotrophic denitrifying reactor (M1) (S^{2-}/NO_3^-)	0 (inoculum)	168.0[b]	√	B
	32	101.0[b]	NA	NA
	67 (pH shock)	15.4[b]	√	C
	102 (recovered pH shock)	ND	√	D
	129	58.6	NA	NA
	164	ND	√	NA
	192	290	NA	NA
Autotrophic denitrifying reactor (M2) (fed with nitrifying effluent)	171 (49)	131.0[b]	√	E
	208 (86)	104.0[b]	√	NA
Nitrifying reactor (NR)	0 (inoculum)	ND	√	NA
	30	98.0[c]	√	NA
	68	ND	√	NA

ND not determined, NA not analyzed, a) activity with $S_2O_3^{2-}$-S, b) activity with S^{2-} and c) activity with NH_4^+-N

Table 7.2 Probes used for fluorescent in situ hybridization and the formamide (FA) concentrations used.

Probe	Probe sequence (5'-3')	FA (%)	Target organisms	Reference
EUB338I	GCT GCC TCC CGT AGG AGT	20	Bacteria domain	[1]
Alf1b	CGT TCG YTC TGA GCC AG	20	*Alphaproteobacteria*	[2]
Bet42a	GCC TTC CCA CTT CGT TT	35	*Betaproteobacteria*	[2]
Gam42a	GCC TTC CCA CAT CGT TT	35	*Gammaproteobacteria*	[2]
Arch915	GTC CTC CCC CGC CAA TTC CT	35	Archaea	[3]
TBD121	CTC GGT ACG TTC CGA CGC	35	*Thiobacillus denitrificans* and *T. thioparus*	[4]
TMD131	TCC CAG TCT TTG AGG TAC	35	*Thiomicrospira denitrificans*	[4]
NSO190	CGA TCC CCT GCT TTT CTC C	55	β-*Proteobacteria* ammonia oxidizing bacteria	[5]
NEU653 (fluos)	CCC CTC TGC TGC ACT CTA	40	Most halophilic and halotolerant *Nitrosomonas* spp.	[6]
Nit3	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.	[7]
CFB560	WCC CTT TAA ACC CAR T	30	β-*Proteobacteria* (*Burkholderiales*)	[8]
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	50	Most members of the phylum *Nitrospirae*	[9]

Reference: [1] Amann *et al.* (1990), [2] Manz *et al.* (1992), [3] Stahl and Amann (1991) [4] Fernández *et al.* (2006), [5] Mobarry *et al*. (1996), [6] Wagner *et al.* (1995), [7] Wagner *et al.* (1996), [8] O´Sullivan *et al*. (2001) [9] Daims *et al*. (2001).

7.4 RESULTS

7.4.1 DGGE profiles of microbial population

Collected samples A, B, C, D and E were analyzed by this technique (Table 7.1). Two denaturing gradients for DGGE were tested with these samples. The gradient composition of the first used gel ranged from 30 to 60% of denaturant and of the second from 40 to 70% (Figure 7.1). The DGGE bands profile of the former showed a lower number of bands with worse resolution than the latter. Therefore, the DGGE gradient from 40 to 70% was selected for further analysis and the most interesting bands from this DGGE gel were excised and sequenced (Figure 7.1 and table 7.3). The 16S sequences obtained were compared to those from the Gen-Bank using Blast and phylogenetic analysis.

Results showed no bands related with *Alphaproteobacteria* throughout the experiment. On the contrary, *Betaproteobacteria* was the dominant population in the studied bioreactor as previously observed in other microbial communities of activated sludge (Bramucci *et al*., 2003). DGGE analysis showed that a species close to *Thiobacillus* sp. with 99% of similarity (Accession number AY578170) was the dominant organism (band 7) throughout the operation (Fig. 7.3; Table 7.3; Table 7.4). This micro-organism is considered the main responsible of the oxidation of reduced sulphur compounds (Robertson and Kuenen, 1992).

Bands 1, 2, 3, 4 and 5, close to different uncultured *Bacteroidetes* spp. with a 99% similarity (Table 7.2), seemed to be characteristic of different stages of the operation (Figure 7.1). Band 1 was detected in the initial inoculum, band 2 and 3 were present along the whole operation but they seem to dominate during the pH shock and recuperation stages respectively, while band 5 seems to be exclusive of the reactor fed with the effluent from the nitrifying reactor. Phylum *Bacteroidetes* is widely distributed in the environment, including soil, seawater, sediments and autotrophic sludge from UASB reactors fed with thiosulphate as electron donor (O`Sullivan *et al*., 2005; Fernández *et al*., 2008)

Band 3 corresponded to phylum *Chlorobi* (FJ710742 with a 95% similarity). This obligatory phototrophic group of bacteria, also known as green sulphur bacteria, is able to

use S^{2-}, S^0, or $S_2O_3^{2-}$ as electron donor for the reduction of CO_2 (Ghosh and Dam, 2009). This micro-organism has been detected in activated sludge and hypersaline wastewater (Bramucci *et al*., 2003; Lefebvre *et al*., 2006).

Band 6 was close to candidate division JS1 (GQ423371) with 94% similarity. This division was discovered in subsea floor sediments by Webster *et al*. (2004) as part of sulphate reducing consortia, in anaerobic methanotrophic communities and tidal flats (Webster *et al.,* 2007). The presence of this microorganism could be justified by the source of inoculum used (anaerobic sludge from a digester treating wastewater from fish canning industry).

When the effluent from the denitrifying reactor was used to feed the nitrifying reactor (sample E) the highest richness was observed. Bands 2, 3, 5, close to *Bacteroidetes* previously detected in freshwater and activated sludge (O'Sullivan *et al*., 2005; Huang *et al*., 2008); band 7 close to *Thiobacillus spp.;* and exclusive bands 8 and 9 were detected. Band 8 was close to *Aquincola* sp. (DQ232437) with 97% similarity. *Aquincola* is a member of *Betaproteobacteria* class. The presence of this micro-organism in the biomass is unclear because *Aquincola* is a new genus able to grow on methyl-*tert*-butyl ether and ethyl-*tert*-butyl-ether which are commonly used as oxygenating compounds in gasoline (Lechner *et al*., 2007, Müller *et al.,* 2008). Band 9 was close to genera *Stenotrophomonas* (FN563156) with 97% similarity. *Stenotrophomonas* is a genus with high catabolic diversity, reported to be responsible of the degradation of different compounds such as phenanthrene, 2,4,6-trinitrotoluene and detergents (Salvadori *et al.,* 2006), and nitrate under semi-anaerobic condition (Yu *et al*., 2009).

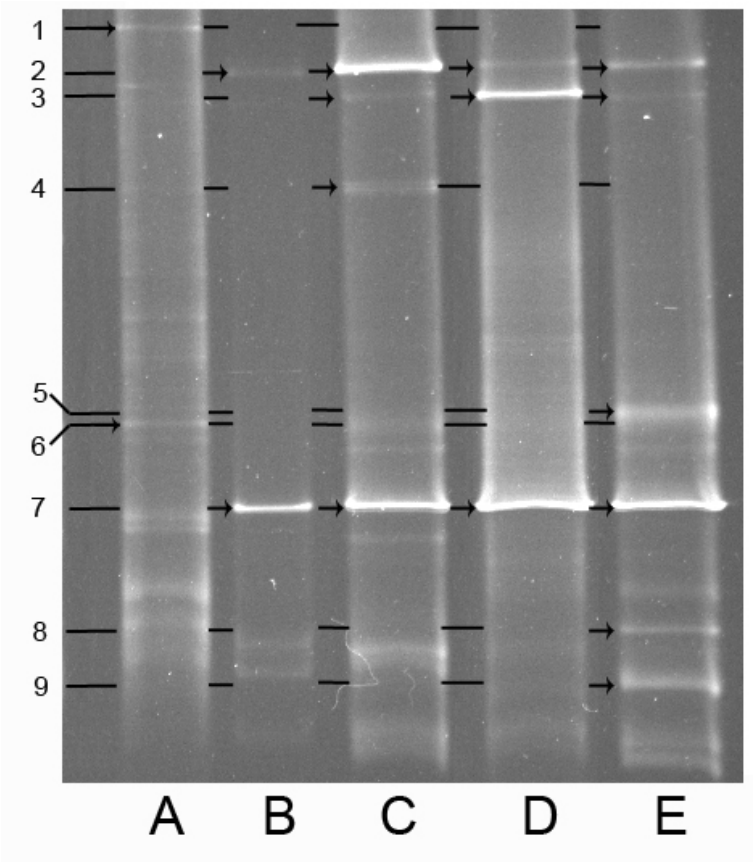

Figure 7.1 DGGE gel at denaturant gradient from 40 to 70% of samples: A) Inoculum of the enrichment reactor (ER); B) Inoculum of the autotrophic denitrifying reactor (M1); C) Operational day 67 (pH shock) of reactor M1; D) Operational day 102 of reactor M1; and E) Sample from autotrophic denitrifying reactor M2 on day 171 of operation.

Table 7.3 Most interesting 16S rRNA gene sequences obtained from DGGE bands (Fig. 7.3) of the different samples. Shown designations and accession numbers for sequences and levels of similarity to related organisms are based on BLAST and a thorough phylogenetic analysis (Fig. 7.4)

DGGE band	Best match	Name	Identities (%)	Phylogenetic group	Closest described
1	DQ660966	Uncultured bacterium isolate DGGE gel band 37	527/528 (99%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; environmental samples	Alphaproteobacteria (DQ660966) (99%)
2	GU062450	Uncultured bacterium clone YC36	532/532 (100%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; environmental samples	Uncultured Bacteroidetes bacterium (100%)
3	FJ710742	Uncultured bacterium clone Dok23	528/528 (100%)	Bacteria; Chlorobi; environmental samples. (95%)	Unc. Chlorobi (95%) (Bacteroidetes/Chlorobi)
4	FN436162	Uncultured bacterium HA||-R60-B-9243-A|	530/534 (99%)	Bacteria; Bacteroidetes; environmental samples	Uncultured Bacteroidetes bacterium (99%)
5	AB241558	*Uncultured bacterium clone e06-a02*	526/528 (99%)	Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; environmental samples	Uncultured Bacteroidetes bacterium (99%)
6	GQ423371	Bacterium enrichment culture clone DGGE-12E	514/523 (98%)	uncultured candidate division JS1 bacterium	uncultured candidate division JS1 bacterium (94%)
7	AY578170	Uncultured Hydrogenophilaceae bacterium	541/543 (99%)	Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae; environmental samples	Thiobacillus sp. (99%)
8	DQ232437	Uncultured bacterium clone CYCW283	540/541 (99%)	Bacteria; Proteobacteria; Betaproteobacteria;	Aquincola (97%)
9	FN533156	Uncultured bacterium HA||-RM37-2-B-6500-J	542/543 (99%)	Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae;	Stenotrophomonas (97%)

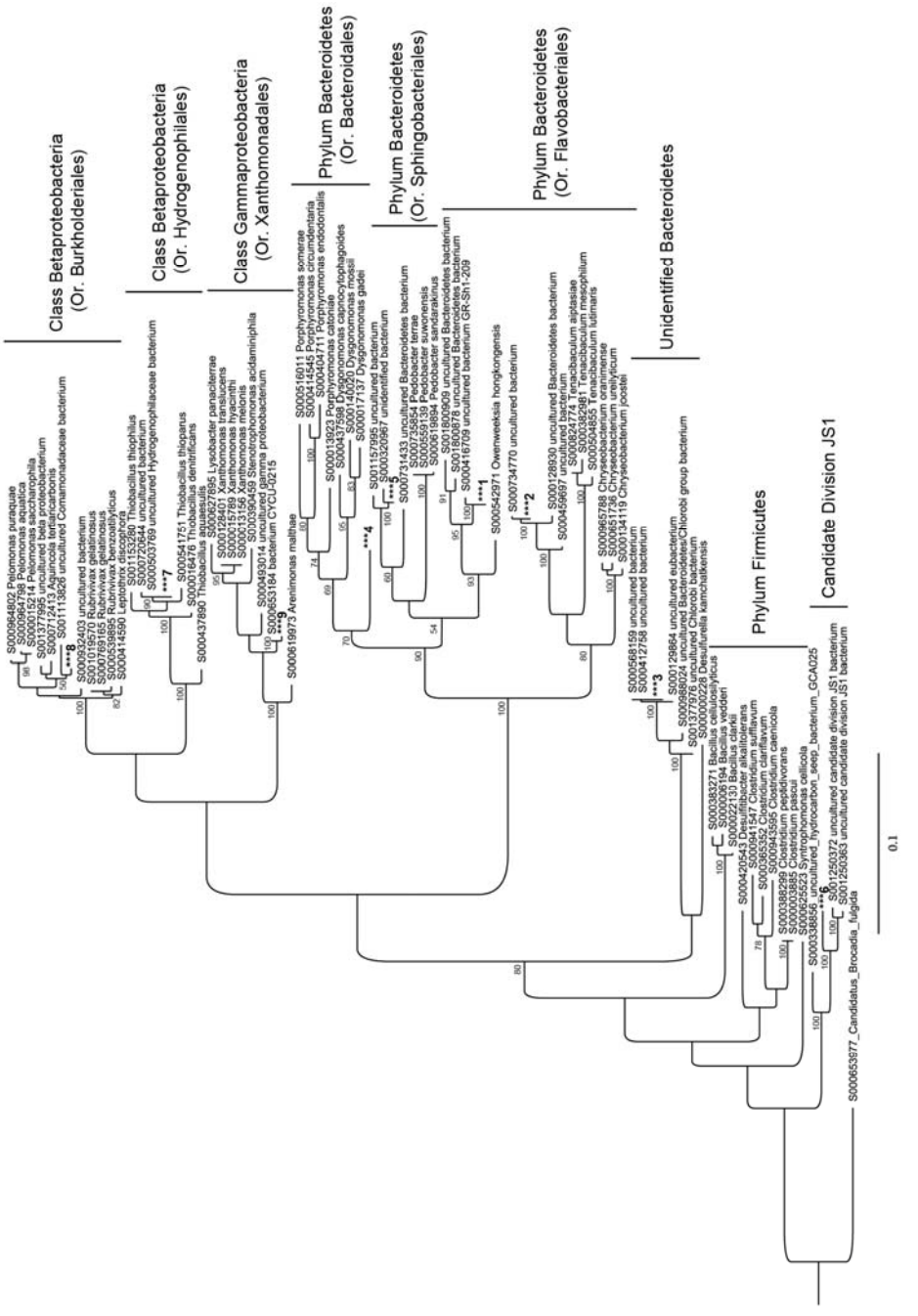

Figure 7.2 Phylogenetic tree of the 16S rRNA sequences obtained from most conspicuous DGGE bands (Fig. 7.3)

7.4.2 Microbial population identification by FISH technique

Samples from different autotrophic reactors were collected as indicated in Table 7.1 and the FISH probes listed in Table 7.2 were assayed.

Autotrophic denitrifying batch reactor (ER)

Anaerobic sludge from an anaerobic digester treating wastewater from a fish canning industry was used as inoculum to produce autotrophic denitrifying biomass fed with a mixture of nitrate and thiosulphate.

The amount of DNA stained with the DAPI dye in the inoculum was poor. Some bacteria gave positive results to probe EUB338I (10%) and some to Gam42a (5%). However, no positive results were detected from the hybridization with general probes Alfb and Bet42a classes (Table 7.4). These results agree with the low specific sulphur oxidizing activity registered (9.5 mg $S \cdot (g\ VSS)^{-1} \cdot d^{-1}$). In previous studies a percentage of 24% of the bacteria in anaerobic sludge have been reported to be affiliated to the phylum *Proteobacteria* with percentages below 5% for each *Alfa-, Beta-, Gamma-* and *Epsilon-proteobacteria* classes (Ariesyady *et al*., 2007).

It should be noted that the presence of inorganic material in the inoculum, which presumably comprises sulphur compounds might serve as support material for the biomass, produced significant autofluorescence which impeded the observation and image acquisition of the samples analyzed with probes containing the fluos and Cy3 dyes.

Table 7.4 Relative percentage of specific microorganism respect to the total bacteria (DAPI) (%)

	Probe			
Sample	**EUB338I**	**Gam42a**	**TBD121**	**NEU653**
Autotrophic denitrifying (Batch reactor) (ER)				
Inoculum anaerobic sludge	10%	5%	0%	0%
Day 60	50%	15%	5%	0%
Day 180	60%	45%	35%	0%
Autotrophic denitrifying reactor (SBR) (M1)				
Inoculum	80%	70%	70%	0%
pH shock (Day 67)	60%	40%	30%	0%
Day 102	60%	50%	40%	0%
Day 164	90%	70%	65%	0%
Autotrophic denitrifying reactor (SBR) Fed with nitrifying effluent (M2)				
Inoculum	90%	75%	70%	0%
Day 171 (49)	90%	75%	70%	2%
Day 208 (86)	90%	75%	70%	2%

After two months of incubation the amount of biomass detected with DAPI significantly increased as the amount of heterogeneous aggregates of short bacillus and rod shaped micro-organisms (Figure 7.3a). Positive result was detected for probe Gam42a (Figure 7.3b) of rod-shaped bacteria. These results agree with those found by Fernández *et al.* (2008) and Haaijer *et al.* (2006) for a culture enriched with thiosulphate and nitrate. In these cases the inorganic material from the samples interfered in the analysis due to its

auto-fluorescence. Some rod shape organism gave positive result for the specific probe TBD121 (*Thiobacillus denitrificans*) (Table 7.4). The specific sulphur oxidizing activity increased from day 37 to 95 from 39 to 187 mg $S \cdot (g\ VSS)^{-1} \cdot d^{-1}$, respectively (see Chapter 3) which confirmed the positive result for the probe TBD121.

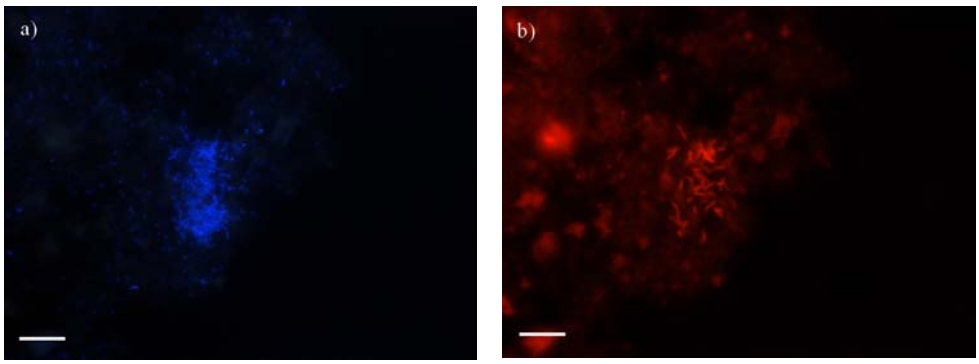

Figure 7.3 Hybridization of biomass enriched on day 60 a) DAPI stain showed heterogeneous biomass, b) Gam42a (Cy3 dye-red) hybridized positively with rod-shaped bacteria (bar 10 µm)

After six months of enrichment the amount of cells in free form detected by DAPI dye remained almost constant and again the presence of inorganic material produced auto-fluorescence that made it difficult to identify the fluorescence produced by positive cells hybridization. Despite the problems in obtaining quality images, the observation under the microscope (Figure 7.4) confirmed the presence of *Thiobacillus denitrificans* and *Gammaproteobacteria,* which is in accordance to Fernández *et al.* (2008).

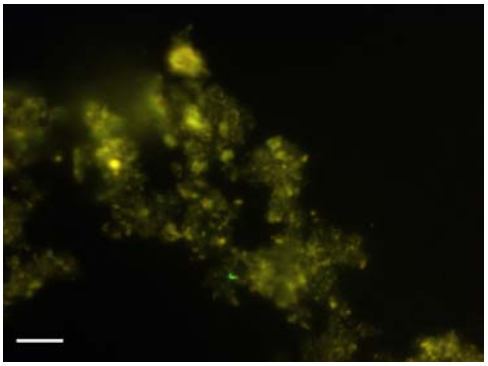

Figure 7.4 This image shows the autofluorescence produced by inorganic material (bar 10 µm).

Continuous Autotrophic denitrifying reactor (SBR) (M1)

For the start-up of the autotrophic denitrifying reactor M1, the enriched culture developed in the previous stage (Chapter 3) was used as inoculum.

The hybridization of enriched biomass, used as inoculum with probes, EUB338I, Gam42a and TBD121 revealed positive results for all probes tested, with percentages of 80%, 70% and 70% respectively with respect to cells stained with DAPI (Table 7.4). The high density in population of *Thiobacillus denitrificans* explains the capacity of the biomass to use sulphide as electron donor. The specific activity of the inoculum was 168 mg S^{2-}·(g VSS)$^{-1}$·d^{-1} (Table 7.1). However, the hybridization with the specific probes TMD131, NEU653 and Ntspa712 gave negative results.

Three our samples of sludge were analyzed during the whole operational period (Table 7.1) and all of them showed the similar responses: positives results with probes EUB338I (60-90 %), TBD121 (30-65 %), Gam42a (40-70 %).

On day 67 of operation the pH increased close to 10. This change affected significantly the population of *Thiobacillus denitricans* and therefore the specific activity of the biomass decreased significantly to values of 15.4 mg S^{2-}·(g VSS)$^{-1}$·d^{-1}. As a consequence the number of cells hybridized with probe TBD121 was low (30%). However,

DGGE analysis showed that the same species close to *Thiobacillus denitrificans* (band 7; Figure 7.1) was still present as dormant cells.

On day 102 (60 days after pH shock), the population of *Thiobacillus* showed a notorious recovery (40% of total biomass hybridized positively with TBD121 probe). DGGE gel identified the same *Thiobacillus* detected previously as dormant cells (Figure 7.1), which confirms that the biomass was able to recover its activity after the pH shock. From this moment on the populations of *Thiobacillus* gradually increased in percentage up to values close to 65%. A similar behaviour was detected for EUB338I and Gam42a probes (Table 7.4)

A particularity of the analyzed samples, observed already in previous works (Fernández *et al*., 2008; Yates *et al*., 2003; Siyambalapitiya and Blackall 2005; Haaijer *et al*., 2006), was detected in this study with regard to the identification of the Thiobacillus denitrificans population. *Thiobacillus denitritificans* and *Gammaproteobacteria* classes were always detected in all analyzed samples. *Thiobacillus denitrificans* is a member of the *Betaproteobacteria* class however no positive results were obtained with Bet42a probe. When a combination of TBD121 and Gam42a probes were applied to the same samples (164 days), positive results were detect for both probes (Figure 7.5). It has been already reported that during the operation of an UASB reactor under autotrophic conditions with thiosulphate as electron donor, the number of *Thiobacillus denitrificans* cells detected was close to that found for *Gammaproteobacteria* cells (Fernández *et al*., 2008).

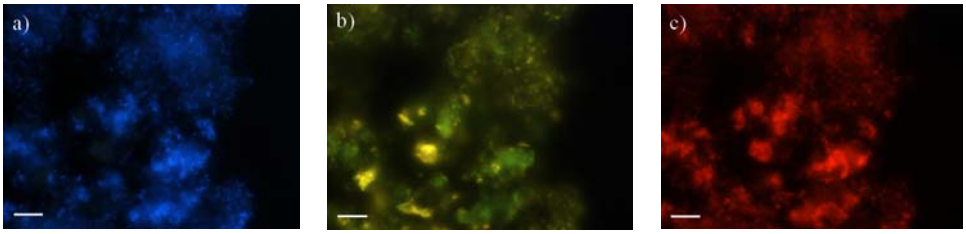

Figure 7.5 Multiple hybridization of a sample collected on day 164 of operation a) DAPI, b) TBD121 (Fluos dye-green) and c) Gam42a (Cy3 dye-red) probes

Several researches have reported that some cells affiliated to *Gammaproteobacteria* were unable to hybridize with Gam42a probe but satisfactorily hybridized with Bet42a (Yates *et al*., 2003; Siyambalapitiya and Blackall 2005). Haaijer *et al*. (2006) reported anomalous hybridization of pure culture of *Thiobacillus denitrificans* (ATCC25259) with Gam42a, being unable to hybridize with Bet42a probe. Cloning and sequencing the 23S rRNA gen of pure culture strain of *Thiobacillus denitrificans* (ATCC25259) they observed the presence of Thymine in position 1033 of the gen sequence, which is in a target site for probe Gam42a (Table 7.5). More studies are necessary to elucidate if the translation of the amino acid Adenine to Thymine present in the *Thiobacillus denitrificans* is a phenomenon exclusive of this species or is generalized at other species of *Thiobacillus*.

Table 7.5 Alignment of the sequence probes Bet 42a, Gam42a and *Thiobacillus denitrificans*

Name	Sequence(5'-3')	Reference
Bet42a	GCC TTC CCA C**T**T CGT TT	Manz *et al*. (1992)
Gam42a	GCC TTC CCA C**A**T CGT TT	Manz *et al*. (1992)
Thiobacillus denitrificans (ATCC25259)	CGG AAG GGT G**T**A GCA AA	Haaijer *et al*. (2006)

Autotrophic denitrifying reactor (SBR) fed with nitrifying effluent (M2)

The composition of the feeding of the previous SBR was changed by a nitrifying effluent. At the initial time the biomass consisted mainly of *Eubacteria*, *Thiobacillus denitrificans* and *Gammaproteobacteria* cells, while negative results for NSO190, NEU653, Nit3 and Ntspa712 probes were registered.

On day 49 of operation (with nitrifying effluent) a sample of biomass was analyzed. Percentages of populations of Eubacteria, *Thiobacillus denitrificans* and Gammaproteobacteria remained constant in the sludge (Table 7.4) according as well to the results obtained by PCR-DGGE (Figure 7.1). However, the specific activity decreased

from 219 mg S^{2-}·(g VSS)$^{-1}$·d^{-1} with synthetic medium to 131 mg S^{2-}·(g VSS)$^{-1}$·d^{-1} when the nitrifying effluent was used as feeding media. This fact indicated that the autotrophic denitrifying activity has been presumably affected by the composition of the feeding. This behaviour has been widely discussed in chapter 5.

In this sample, small aggregates of biomass gave positive results for NSO190 probe, which indicated that some ammonium oxidizing bacteria coming with the effluent from the nitrifying reactor survive under autotrophic conditions.

Taking into account this fact, the specific probe for *Nitrosomonas sp*. (NEU653) and TBD121 were tested in the sample corresponding at 208 days of operation. Positive results were obtained and *Nitrosomonas spp*. could be detected in populations in the form of small aggregates (Figure 7.6).

The microorganism that hybridized positively with NEU653 might have survived with residual ammonium from the nitrifying effluent. Similar behaviour has been previously reported in a SHARON/Anammox system where ammonium oxidizing activity was detected in the Anammox biomass (Dapena-Mora *et al*., 2006).

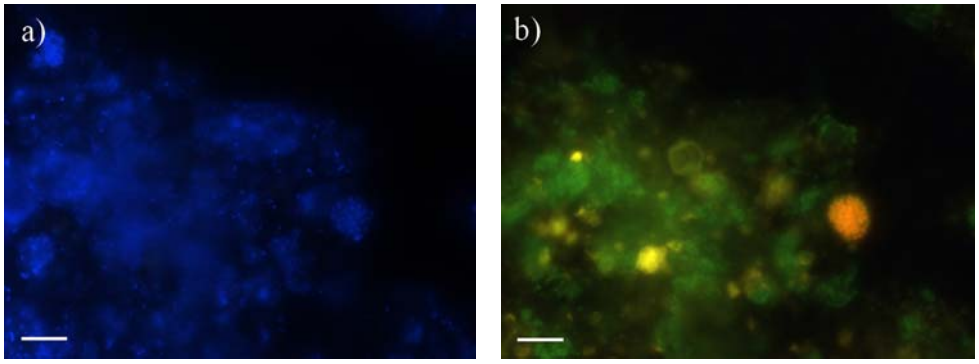

Figure 7.6 a) Image of the total DNA (DAPI blue) and b) simultaneous hybridization for TBD121 (Fluos dye-green) and NEU653 (Cy3 dye-orange).

The relative amount of microbial population remained constant during whole operation being *Thiobacillus denitrificans* the dominant population. These observations

agree with the high sulphide removal efficiencies detected during the whole operational period.

Nitrifying Reactor (NR)

For the characterization of the microbial community from the nitrifying reactor initially the general probes (EUB338I, Alf1b, Bet32a, and Gam42a) were applied to detect the main bacterial groups present in the sludge. Then specific probes for ammonium oxidizing (NSO190, NEU653) and nitrite oxidizing bacteria (Nit3, Ntspa712) were applied.

Hybridization of inoculum showed positive results to probe EUB338I and some members of *Betaproteobacteria* class, while no positive results were obtained for probes NSO190, NEU653 and Nit3.

On day 30 of operation (stationary state conditions), the amount of *Betaproteobacteria* class was close to the total of Eubacteria amount (EUB338I) and positive results for NSO190 (*Betaproteobacteria* ammonia-oxidizing bacteria) were registered. At this moment, high efficiencies of ammonium oxidation were measured of 350 mg NH_4^+-N (g VSS)$^{-1}\cdot d^{-1}$, suggesting the presence of some specie of *Nitrosomonas*. This fact, was confirmed when the specific probe for *Nitrosomonas* (NEU653) was applied and positive results were obtained (Figure 7.7b in green).

The low concentration of nitrite in the effluent suggested the activity of nitrite oxidizing bacteria. In order to confirm this observation. On day 68 of operation the specific probe for *Nitrospira sp.* (Ntspa712) was applied obtaining positive results (Figure 7.7b). The presence of this populations has been already reported in granular sludge systems from a SBR reactor for the post-treatment of the effluent form an anaerobic digester treating the wastewater produced in a fish canning industry (Figueroa *et al.,* 2008).

The populations distribution observed during whole operational period remained almost constant.

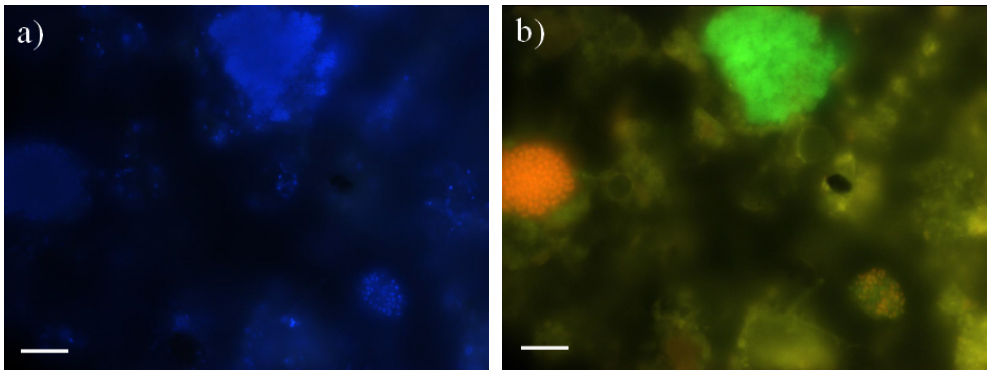

Figure 7.7 Biomass sample on day 68 of operation. a) Total biomass (DAPI). b) *Nitrosomonas sp*. (NEU653) (green) and *Nitrospira sp*. (Ntspa712) (orange) (bar 10 µm).

7.5 CONCLUSIONS

The application of the FISH technique to biomass collected from autotrophic denitrifying reactors allowed the identification of populations able to use sulphur compounds corresponding mainly to *Thiobacillus denitrificans*. Changes in pH values played an important role over this population. The increase of this parameter originated a decrease on the specific autotrophic activity of the biomass which was correlated to a diminution of the percentage of *Thiobacillus denitrificans* in the analyzed sample. Furthermore the population of *Thiobacillus denitrificans* remained constant even when the feeding was switched by the effluent of the nitrifying reactor.

The study of the phylogenetic composition of the biomass gave as results that the predominant population in all samples analyzed with exception of the inoculum was *Thiobacillus denitrificans* with a 99% similarity. Another representative group detected almost in all samples was close to *Bacteroidetes* with a 99% similarity. This group is widely distributed in the environment including soil, seawater, sediment and some kind of reactors as UASB.

Surprisingly another two groups able to use a sulphur source were detected one close to phylum *Chlorobi* and another close to candidate division JS1 with 95% and 94% similarity, respectively.

The sample collected from the Nitrifying/Autotrophic Denitrifying reactor revealed the presence of microorganisms close to *Stenotrophomonas* with 97% similarity. It has been reported that some members of these genera can use nitrate under semi-anaerobic condition.

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Conclusions

The main conclusions of this research, which was focused on the development of autotrophic denitrifying biomass and the operation of a sequential batch reactor for treatment of wastewater containing high concentration of sulphide and nitrate, are now presented.

1. Enrichment of Autotrophic denitrifying biomass

The batch system was a good tool to develop autotrophic denitrifying biomass, which presented appropriate characteristic of VSS = 7.8 $g \cdot L^{-1}$, VSS/TSS ratio = 0.54 $g \cdot g^{-1}$ and SVI= 70.2 $mL \cdot (g\ VSS)^{-1}$ and was able to use different sulphur source to carry out the autotrophic denitrification. The maximum specific autotrophic denitrifying activity (ADA) was observed with thiosulphate as electron donor (216 mg NO_3^--$N \cdot (g\ VSS)^{-1} \cdot d^{-1}$). When sulphide was used, the specific ADA was significantly reduced and influenced by S/N ratio (153 mg NO_3^--$N \cdot (g\ VSS)^{-1} \cdot d^{-1}$ at S^{2-}/N of 0.35 $g \cdot g^{-1}$). The specific ADA with elemental sulphur was not conclusive due to the fact that the value of specific ADA of the control without external sulphur (12.5 mg NO_3^--$N \cdot (g\ VSS)^{-1} \cdot d^{-1}$) was similar compared to the value obtained in the presence of S^o (11.5 mg NO_3^--$N \cdot (g\ VSS)^{-1} \cdot d^{-1}$). The presence of a sulphur source stored in the sludge was confirmed and a value of specific ADA of 121 NO_3^--$N \cdot (g\ VSS)^{-1} \cdot d^{-1}$ was measured in absence of sulphur source.

2. Characterization of population of Autotrophic denitrifying biomass

The FISH technique revealed that the microbial communities present in the enriched biomass and reactors were characterized by *Thiobacillus denitrificans* even when the feeding was switched to the effluent of the nitrifying reactor. pH values played an important role on this population and an increase of this parameter originated a decrease on the percentage of *Thiobacillus denitrificans* biomass.

The study of the phylogenetic composition of the biomass showed that the predominant population was *Thiobacillus denitrificans* with a 99% similarity. Another representative group detected was close to *Bacteroidetes* with a 99% similarity. Two groups able to use a sulphur source were detected, one close to phylum *Chlorobi* and

another close to candidate division JS1 with 95% and 94% similarity, respectively. Finally, one group able to use nitrate under semi-anaerobic condition was close to *Stenotrophomonas* with 97% similarity.

3. Kinetic properties of autotrophic denitrifying biomass

The kinetic parameters obtained for autotrophic denitrifying biomass using sulphide as electron donor are a useful tool for the design and operation of autotrophic denitrifying reactors. Under stoichiometric conditions, the maximum nitrate concentration which can be treated does not exceed the K*i* (355 ± 4.38 mg NO_3^--N·L^{-1}) and the maximum nitrite concentration does not exceed the IC_{50} value (48 mg NO_2^--N·L^{-1}). A control of nitrous oxide production should be maintained to guarantee the efficiency of autotrophic denitrification.

4. Operation of SBR reactors

The SBR is a suitable technology to carry out the autotrophic denitrification of wastewaters containing sulphide and nitrate. It was able to treat loading rates of 0.45 g NO_3^--N·L^{-1}·d^{-1} and 0.45 g S^{2-}·L^{-1}·d^{-1} with removal efficiencies around 67% and 100%, respectively. Control of pH is an important parameter to ensure optimum conditions for microbial activity.

The combination of nitrification/autotrophic denitrification could be a possibility for the postreatment of fish cannery effluents. The operational costs are lower than those of the conventional nitrification/denitrification processes.

List of Acronyms

ANAMMOX	ANaerobic AMMonium OXidation
ANANOX	ANaerobic ANoxic OXic
ADA	Autotrophic Denitrifying Activity
ALR	Ammonium Loading Rate
COD	Chemical Oxygen Demand
CSRT	Continuous Stirred Tank Reactor
Cy3	Iodocarbocyanide
DAPI	4,6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
EGSB	Expanded Granular Sludge Bed
EUB338I	*Eubacteria* domain
FISH	Fluorescence In Situ Hybridization
FLUOS	5,6 carbyfluorescein-N-hydroxysuccinimide ester
Gam42a	*Gammaproteobacteria* class
GC	Gas Chromatography
GP	Gs Phase
GPV	Gas Phase Vial
HRT	Hydraulic Retention Time
IC	Inorganic Carbon
LPR	Liquid Phase Reactor
LPV	Liquid Phase Vial
NEU653	*Nitrosomonas spp.*
NLR	Nitrogen Loading Rate
NSO190	*Betaproteobacteria* ammonia oxidizing bacteria
Ntspa712	*Nitrospirae phylum*
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PLC	Programmable Logic Controller
SBR	Sequencing Batch Reactor
SOA	Sulphur Oxidizing Activity
SHARON	Single reactor High Ammonia Removal Over Nitrite
SLAD	Sulphur Limestone Autotrophic Denitrification
SLR	Sulphide Loading Rate

SNLR	Specific Nitrogen Loading Rate
SRT	Solid Retention Time
SOB	Sulphur Oxidizing Bacteria
SVI	Sludge Volumetric Index
T	Temperature
TBD121	*Thiobacillus denitrificans*
TC	Total Carbon
TOC	Total Organic Carbon
TMD131	*Thiomicrospira denitrificans*
TSS	Total Suspend Solids
UASB	Up flow Anaerobic Sludge Bed
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

List of Publications

Articles

Fajardo C., Mosquera-Corral a., Campos J.L. y Méndez R. (2007). Depuración Conjunta de Aguas residuales Ricas en Nitratos y Efluentes con Compuestos Reducidos del Azufre. Revista Técnica del Medio Ambiente RETEMA, No **127**: 39-51. **ISSN 1130-9881**

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