ESTUDIO DE LA SULFOXIDACION ORGANO-LITOTROFICA Y LITOTROFICA BAJO CONDICIONES DESNITRIFICANTES

Tesis que para obtener el grado de

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STUDY OF ORGANO-LITHOTROPHIC AND LITHOTROPHIC SULFO-OXIDATION UNDER DENITRIFYING CONDITIONS

PhD Thesis

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I would like to dedicate this document to my family, my new family and mainly to my princess, Gehovana.

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The important thing is not to stop questioning. –

Albert Einstein

CONTENTS

RESUMEN

Las descargas de aguas residuales provenientes de la Industria Petroquímica tienen gran relevancia a nivel mundial, por la presencia de compuestos aromáticos, amonio y altas concentraciones de sulfuro principalmente; los cuales tienen un impacto adverso en los ecosistemas naturales y en la salud pública. Debido a esto, cobra importancia la necesidad de desarrollar y caracterizar sistemas biológicos que permitan que las descargas de aguas residuales no causen un deterioro a los cuerpos receptores de agua.

En este trabajo se estudió la desnitrificación organo-litotrófica y litotrófica, tanto en cultivos en lote como en continuo. Para el estudio de la desnitrificación organo-litotrófica, el lodo desnitrificante utilizado para los cultivos en lote y en continuo fue obtenido de un reactor UASB, el cual tuvo eficiencias de consumo de sulfuro, fenol y nitrato de 100%.

En cultivos en lote se observó una oxidación completa de sulfuro y fenol acoplado a la reducción de nitrato a N_2, cuando el nitrato se alimentó a concentraciones estequiométricas. La oxidación de sulfuro y fenol fue secuencial, primero el sulfuro se oxidó a sulfato, y consecutivamente el fenol se consumió. Las velocidades de reacción encontradas fueron: $q S^{2-} > q S^0 = q SO_4^{2-} > q$ fenol. Cuando la concentración inicial de sulfuro se incrementó de 20 a 26 y 32 mg/L la velocidad de oxidación del sulfuro no se afectó significativamente, mientras que el metabolismo desnitrificante para la oxidación de fenol se vio afectado, con una acumulación de N_2O y una disminución en la velocidad de consumo de fenol y nitrato.

Con la finalidad de estudiar el consumo de fenol y sulfuro en continuo, la oxidación simultánea de fenol y sulfuro se evaluó a diferentes velocidades de carga utilizando un reactor de lecho fluidificado inverso desnitrificante. Las velocidades de carga alcanzadas fueron de 167 mg C-fenol/L-d, 37 mg S^{2-}/L-d y 168 mg N-NO_3^-/L-d a un TRH de 0.9 d. En el estado estacionario desnitrificante las eficiencias de consumo de fenol, sulfuro y nitrato fueron de 100%, alcanzando rendimientos de producto de 0.82 ± 0.04 C-inorgánico/g C-fenol consumido, 0.99 ± 0.04 g S-SO_4^{2-}/g S^{2-} consumido y 0.89 ± 0.06 g N_2/g N-NO_3^- consumido. El análisis molecular del 16S rDNA en la biopelícula desnitrificante mostró algunas bacterias tales como *Thiobacillus denitrificans*, *Thiobacillus sp*., y *T. sajanensis,* las cuales tal vez pudieron haber participado en el consumo de fenol y/o sulfuro. Estos son los primeros resultados donde se evidencia la oxidación simultánea de fenol y sulfuro empleando un reactor de biopelícula desnitrificante.

Hay escasas evidencias en un reactor continuo de tanque agitado alimentado con acetato, sulfuro y nitrato, donde los productos finales fueron CO_2, S^0 y N_2. En el presente trabajo siendo utilizado el fenol en vez de acetato, el sulfuro se oxidó principalmente a sulfato. Por lo tanto, se emplearon cultivos en lote para investigar la cinética de la desnitrificación litotrófica acoplada a la oxidación de S^0, $S_2O_3^{2-}$ y S^{2-}. Los resultados mostraron que la velocidad de desnitrificación se afectó por el tipo de compuesto azufrado. El orden de las velocidades de reacción fueron: $S_2O_3^{2-} > S^{2-} > S^0$. También se investigó el efecto de la concentración inicial de S^0, sulfuro y nitrato sobre la cinética de la desnitrificación litotrófica. La velocidad de desnitrificación incrementó con la concentración inicial de S^0. No obstante, la concentración inicial de sulfuro tuvo un efecto inhibitorio en la velocidad de desnitrificación. Cuando se probaron diferentes concentraciones iniciales de nitrato, se observó que la relación molar S^{2-}/NO_3^- influyó en el producto final formado durante la oxidación del sulfuro. A una concentración sub-estequiométrica de nitrato se promovió la formación de S^0 en vez de sulfato.

A pesar de que el S^0 tiene baja solubilidad, en los experimentos previos se evidenció que al incrementar la concentración inicial de S^0 se incrementó la velocidad de desnitrificación. Considerando esto, se evaluó como una posible tecnología alternativa un reactor de lecho empacado con S^0-limestone para eliminar el nitrato. El reactor de lecho empacado después de 30d de operación y a un TRH de 15h, alcanzó una velocidad de carga de 161 mg N-NO_3^-/L-d. La eficiencia de consumo de nitrato fue de 100%, siendo el nitrógeno molecular el producto final. Las concentraciones de nitrato probadas en este trabajo son concentraciones que típicamente podemos encontrar en las aguas subterráneas (18 mg N- NO_3^-/L).

Utilizando la información de este trabajo, junto con lo reportado en la literatura, en la sección de recomendaciones se presenta una posible propuesta para tratar el agua residual de la Industria Petroquímica.

SUMMARY

The discharges of wastewaters coming from the petrochemical industry have great relevance world wide, due to the presence of aromatic compounds, ammonium and high concentrations of sulfide mainly, which have an adverse impact in natural ecosystems and public health. Thus, it is necessary to find and to characterize biological systems that remove compounds from residual waters discharges avoiding further deterioration to the water bodies.

In this work the organo-lithotrophic and lithotrophic denitrification were studied either batch or continuous culture. For the study of organo-lithotrophic denitrification, the denitrifying sludge used for inoculating batch and continuous cultures was obtained from a UASB reactor, which had efficiencies of sulfide, phenol and nitrate consumption of 100%.

In batch cultures it was observed a complete oxidation of sulfide and phenol which was coupled to the nitrate reduction when this was supplemented at stoichiometric concentrations. The sulfide and phenol oxidation was sequential, first sulfide was oxidized to sulfate, and phenol was successively consumed. The reactions rates found were: $qS^{2-} > qS^{0} = qSO_4^{2-} > q$ phenol. When initial sulfide concentration was increased from 20 to 26 and 32 mg/L the specific rates of sulfide oxidation were not significantly affected, while denitrifying metabolism for phenol oxidation was affected accumulating N_2O. It was also observed a diminishing on specific rates of phenol and nitrate consumption.

In order to study the phenol and sulfide consumption in continuous mode, the simultaneous phenol and sulfide oxidation at different loading rates was evaluated employing a denitrifying inverse fluidized bed reactor. The phenol, sulfide and nitrate loading rates achieved were 167 mg phenol-C/L-d, 37 mg S^{2-}/L-d and 168 mg NO_3^--N/L-d at HRT of 0.9 d. Consumption efficiencies in the steady state of phenol, sulfide and nitrate were 100%, reaching an inorganic carbon yield of 0.82 g/g phenol-C consumed, sulfate yield of 0.99 ± 0.04 g SO_4^{2-}-S/g sulfide consumed and molecular nitrogen yield of 0.89 ± 0.06 g N_2/g NO_3^--N consumed. When microbial analyze of 16S rDNA on denitrifying biofilm was made, it was observed some microorganisms such as *Thiobacillus denitrificans*, *Thiobacillus sp*. and *T. sajanensis*, which possibly participated in the sulfide and phenol consumption. These are the first results where is shown the simultaneous phenol and sulfide oxidation using a denitrifying biofilm reactor.

There are few evidences in a continuous stirrer tank reactor fed with sulfide, nitrate and acetate where S^0, N_2 and CO_2 were the end products. In the present work being phenol instead of acetate, sulfide was mainly oxidized to SO_4^{2-} and the S^0 concentration was very low. Thus, batch cultures mode was employed to investigate the kinetics of lithotrophic denitrification linked to S^0, thiosulfate and sulfide. The results showed that denitrification rates were affected by the type of sulfur compound. It was seen that the order of the reaction rates found were: $S_2O_3^{2-} > S^{2-} > S^0$. Also was investigated the effect of S^0, sulfide and nitrate initial concentration on the kinetics of lithotrophic denitrification. The rate of denitrification increased with S^0 concentration. Nonetheless, the initial sulfide concentration had an inhibitory effect on the denitrification rates. When different nitrate initial concentrations were tested, it was observed that the S^{2-}/NO_3^- molar ratio influenced in the type of final product during the sulfide oxidation. A substoichiometric concentration of nitrate could be used to promote partial oxidation to S^0, as no sulfate was produced.

Although the S^0 has low solubility, in the previous experiments was evidenced that increasing the S^0 concentration, the denitrification rates increased. Considering this, it was evaluated as a possible technological alternative a packed bed reactor with an ratio 1:1 of sufur:limestone granules for removing nitrate. The packed bed reactor after 30 d and with 15 h as hydraulic retention time reached a removal rate of 161 mg NO_3^--N /L-d. The consumption efficiency of nitrate was 100%, being the end product molecular nitrogen. The nitrate concentrations tested in this work were concentrations typically found in the groundwater (18 mg NO_3^--N /L).

Using the information of this work, together with that found in the literature, in chapter of recommendation is presented a possible proposal for the wastewater treatment from the petrochemical industry.

CHAPTER 1

INTRODUCTION

At present, the contamination by carbon-, nitrogen- and sulfur-containing compounds in wastewaters and water bodies is a serious environmental problem. Some wastewaters as those from the oil industry (spent caustic and sour water) represent a challenge for treatment before discharge because of its chemical complexity. These effluents may contain a high concentration of organic compounds such as phenol, ammonia and sulfide that can be removed by biological processes (Olmos *et al.,* 2004). There are evidences suggesting that phenolic compounds are toxic, carcinogenic and mutagenic (Autenrieth *et al.,* 1991). Thus, the removal of aromatic compounds by microorganisms is an essential contribution to the global carbon cycle as well as to the detoxification of wastewaters and contaminated soils (Philipp and Schink, 2000). The key to the evaluation of the fate of organic chemicals in the environment is a realistic evaluation of their susceptibility to mineralization (the oxidation to carbon dioxide). The majority of these compounds (phenol, cresols, xylene, toluene, etc.) can be used as carbon and energy sources for several microorganisms.

The increase of anthropogenic activities has contributed to local unbalances in the natural sulfur cycle, leading to several serious environmental problems: acid rain, odor nuisance from polluted rivers, landfills or treatment systems, corrosion, heavy metal and sulfuric acid release from oxygen exposed mineral ores and soils. Industrial wastewaters containing sulfur compounds also contribute to the sulfur unbalance (Colleran *et al.,* 1995; Lens *et al.,* 1998). Sulfide containing waste streams are generally treated by chemical methods which involve high chemical and disposal costs (Butler and Nadan, 1981; Cadena and Peters, 1988). The ability of autotrophic bacteria to oxidize sulfide at high rates has led to the development of biotechnological methods to remove sulfide from wastewater (Sublette and Sylvester, 1987; Gadre 1989; Kim *et al.,* 1990). There are several studies that show the inhibition of sulfide for many microorganisms, nevertheless, there are only few studies that quantitatively report the sulfide inhibition kinetics (Okabe *et al*., 1995; Maillacheruvu and Parkin, 1996). The inhibitory effect of sulfide is presumed to be caused by unionized hydrogen sulfide, because only neutral molecules permeate well the cell membrane (Reis *et al*., 1992; Oude Elferink *et al*., 1994). Hao *et al.* (1996) proposed that sulfide combines with the iron of ferredoxin and cytochrome in the cell, stopping the electron transport system. In the anaerobic wastewater treatment reactors the inhibitory effects for mixed cultures vary widely. The observations from several studies showed that the toxicity of sulfide depends on the following factors: carbon/sulfur ratio, type of carbon source, type of bacteria (attached or suspended), concentration of a given

sulfide species, and age of culture (Postgate, 1984; Hilton and Oleszkiewicz, 1987; Hilton and Oleszkiewicz, 1988).

On the other hand, wastewater streams containing nitrogen compounds may cause serious environmental problems if these compounds are not properly removed before discharge into the receiving water bodies. A too high nitrogen concentration in the receiving waters can lead to eutrophication, i.e. algal outbreaks and/or fish death in rivers, lakes, and coastal areas (Metcalf and Eddy, 1991; Mussati *et al.,* 2002). Denitrification is a biological process involved in many engineering applications including nitrogen removal from wastewater and groundwater (Hiscock *et al.,* 1991). The need to eliminate aromatic, sulfur and nitrogen compounds has led to the development of new biotechnologies for the detoxification and elimination of the pollutants rather than the conventional approach of disposal.

I. Biotransformation of nitrogen compounds

In order to protect lakes and other natural water bodies from eutrophication, stringent nutrient level is set for the effluent from the wastewater treatment plants (Peng and Zhu, 2006). Because biological nitrogen removal can be effective and inexpensive, it has been widely adopted instead of the physical-chemical processes (EPA, 1993). Various novel biological nitrogen removal processes such as short-cut nitrification and denitrification, anaerobic ammonium oxidation (ANAMMOX) process, completely autotrophic nitrogen removal over nitrite (CANON) process and oxygen-limited autotrophic nitrification-denitrification (OLAND) process, have been proposed (Verstraete and Philips, 1998). These processes involved biochemical transformations of nitrogen compounds catalyzed by enzymes that require metals as cofactors. The metals most important in these processes are iron, molybdenum and copper. The availability and distribution of these metals may have an effect upon the cycling of nitrogen in both fixation and formation of molecular nitrogen.

The nitrogen cycle is composed of four major biologically mediated processes that control the redox state of nitrogen. These processes shown in Figure 1 are: nitrogen fixation, ammonification, nitrification and denitrification (Caumette *et al.,* 1996). The major redox states and nitrogen compounds involved are -3, -1, 0, +1, +2, +3, +5 for ammonia (NH_3), hydroxylamine (NH_2OH), molecular nitrogen (N_2), nitrous oxide (N_2O), nitric oxide (NO), nitrite (NO_2^-), and nitrate (NO_3^-), respectively. For the four processes, nitrogen fixation, ammonification and denitrification are reductive processes while nitrification is an oxidative process. Three of the compounds involved,

ammonia, nitrite, and nitrate, can be taken up for biological use in proteins and nucleotides. The biochemical capability of the organisms to transform N_2 into these chemical forms is vital to life on earth.

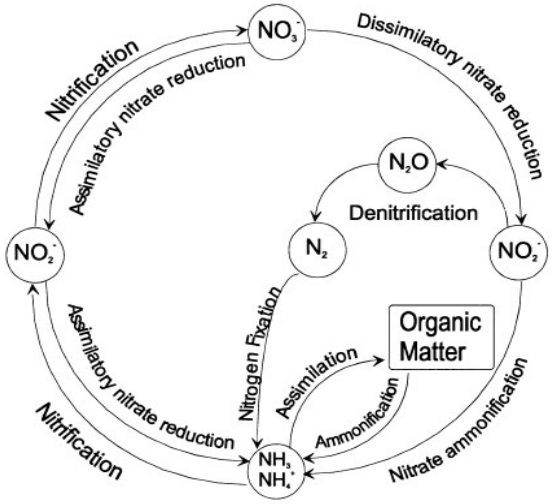

Figure 1 The biogeochemical cycling of nitrogen (Caumette *et al.,* 1996).

II. Nitrification process

The oxidation of ammonium plays a pivotal role in generating a source of nitrate for denitrifying bacteria. The coupling of this oxic process (nitrification) with an anoxic process (denitrification) leads to the releasing of nitrous oxide and/or molecular nitrogen to the atmosphere (Herbert, 1999). Nitrification is the metabolic pathway where the ammonia is oxidized to nitrite and nitrate by lithoautotrophic ammonia and nitrite oxidizing bacteria. The nitrifying bacterium *Nitrosomonas europaea* can obtain all its carbon for growth from CO_2 and all its energy for growth from the oxidation of NH_3 and is considered an obligate chemolithoautotrophic genus. These nitrifying bacteria are sensitive to the presence of organic matter that can inhibit the nitrification process (Pantea-Kiser *et al.,* 1990). Nevertheless, taking in account the complete genomic sequence of *Nitrosomonas europaea* which revealed a potential permease for fructose, Hommes *et al.* (2003) showed that *N. europaea* could use fructose as carbon source to support growth, although the microbial growth rate is very slow indeed. As well, it was observed that the nitrification is possible in presence of low concentration of aromatic compounds, such as *p*-cresol, benzene, toluene and *m*-xylene (Texier and Gómez 2002; Zepeda *et al.,* 2003, 2006, 2007).

Environmental factors are important in regulating nitrifying activity. These include temperature, NH_4^+ and O_2 concentration, pH, dissolved CO_2, salinity, presence of inhibitory compounds, initial concentration of biomass, type and history of the inoculum, among other factors (Herbert, 1999). The free energy of the reaction for ammonia oxidation to nitrate shows that it is exergonic.

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \qquad \Delta G^{\circ\prime} = -351 \text{ kJ/reaction}$$

Most of the data reported in the literature are derived from axenic culture studies and therefore extrapolation of how these parameters affect *in situ* nitrifying activity must be interpreted with care (Herbert, 1999).

III. Denitrification process

Nitrate coming from nitrification process can be removed through the anoxic denitrification process, which is the anaerobic microbial process in which nitrate (NO_3^-) is reduced into molecular nitrogen (N_2). Denitrification involves four enzymatic steps via the intermediates nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (Zumft, 1997), and it is accomplished with a variety of electron donors, including easy consumption compounds such as methanol, acetate, ethanol, lactate and glucose (Grabinska-Loniewska, 1991; Tam *et al.,* 1992; Akunna *et al.,* 1993). Nevertheless, chemical compounds like aromatic hydrocarbons or phenolic compounds can also be used. The enzymes necessaries for the complete nitrate reduction have been identified in different genera of facultative respiration, such as *Parococcus denitrificans*, *Pseudomonas stutzery*, *Escherichia coli*, and *Thiosphaera panthotropha* (Baumann *et al.,* 1996).

The initial step in denitrification is catalyzed by the nitrate reductase. This enzyme is a terminal component in the electron transport chain and its synthesis is thought to be induced by the presence of nitrate and the absence of oxygen (Tiedje *et al.,* 1974; Payne 1981; Stewart 1988). Oxygen suppresses denitrification (Bryan, 1981; Gayon and Dupetit, 1982; Hochstein *et al.,* 1984) and the synthesis and/or activity of denitrifying enzymes (Showe and DeMoss, 1968; Kobayashi and Ishimoto, 1973; Kapralek *et al.,* 1982). Van`t Riet *et al.* (1968) showed that the oxygen affected both the synthesis and activity of nitrate reductase. Krul and Veeningen (1977) observed that synthesis of nitrate reductase by bacteria isolated from activated sludge declined significantly with increasing

oxygen concentration at levels greater than 4 mg/L. Korner and Zumft (1989) determined that the threshold oxygen level which avoided enzyme synthesis varied for each of the three denitrifying enzymes. The maximum oxygen concentrations that would allow the synthesis of nitrate reductase, nitrite reductase and nitrous oxide reductase were 5.1, 2.5 and 3.8 mg/L, respectively. Bonin *et al.* (1989) observed that the enzymes associated with denitrification were affected differently with respect to oxygen concentration. Nitrate reductase was less sensitive towards oxygen than nitrite and nitrous oxide reductases. Nitrate reductase activity was completely blocked at an oxygen concentration greater than 4.05 mg/L, compared with 2.15 and 0.25 mg/L for nitrite and nitrous oxide reductases, respectively.

The inhibition or the blockade of synthesis of nitrate reductase by oxygen presence is still not clear, however, there are some hypotheses about this. For example, Noji and Tanigushi (1987) concluded that oxygen blocks the ability of nitrate to reach the active site of nitrate reductase. Hackett and MacGregor (1981) proposed that oxygen avoided the incorporation of nitrate reductase into the cytoplasmic membrane. Kapralek *et al.* (1982) reported that oxygen blocks the synthesis of nitrate reductase at the level of transcription. However, Hernandez and Rowe (1988) demonstrated that oxygen inhibits nitrate uptake instead of nitrate reduction in 11 out of 12 species tested. Nitrate transport by whole cell suspensions was completely and reversibly inhibited, whereas nitrate reduction by cell-free extracts was not affected by oxygen or was only partially inhibited in some cases.

Finally, the denitrification process can be affected by several factors, being the end products NO_2^-, NO and N_2O, which are undesirable compounds for achieving a feasible system. The accumulation of nitrite can inhibit the nitrate consumption. Klotter (1969) reported that, at a high concentration of nitrite (above 30 mg NO_2^--N/ L), the nitrate consumption was inhibited. Almeida *et al.* (1994) also showed that the denitrification process was inhibited by nitrite concentration of 66 µg N/L. On the other hand, the effect of NO and N_2O on denitrification process has not been reported. The main factors affecting the accumulation of intermediates in denitrification are: oxygen concentration, C/N molar ratio, nitrite concentration and pH (Anderson and Levine, 1986; Hong *et al.,* 1994; Thomsen *et al.,* 1994; Cervantes *et al.,* 1998).

III. 1 Lithotrophic denitrification

The denitrification process can be lithotrophic or organotrophic depending on the energy source. Nevertheless, it is possible to have an organo-lithotrophic denitrification where both organic and inorganic compounds are used as energy sources (Reyes-Avila *et al.,* 2004). In the lithotrophic denitrification, reduced inorganic sulfur compounds can be used as energy source, while the carbon source is carbon dioxide, which is fixed through the Benson-Calvin cycle. Several chemolithoautotrophic bacteria have the metabolic capability to anaerobically oxidize reduced inorganic sulfur compounds, such as sulfide (S^{2-}), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), or sulfite (SO_3^{2-}), at the expense of the reduction of nitrate or other oxidized nitrogen compounds such as NO_2^- and N_2O (Schedel and Truper, 1980; Timmer-ten Hoor, 1981; Kuenen *et al.,* 1992). Sulfur-utilizing chemolithoautotrophic denitrifiers are believed to play an important role in mineral cycling by linking sulfur and nitrogen cycles (Sørensen, 1987; Korom, 1992), among these, pure cultures such as the well known obligate autotrophic *Thiobacillus denitrificans* (Timmer-Ten Hoor, 1975; Schedel and Truper, 1980; Kuenen *et al.,* 1992), and microbial consortia (Reyes-Avila *et al.,* 2004). *T. denitrificans* is distinguished from all other *Thiobacillus* species by its ability to grow as facultative anaerobic chemolithotroph, coupling the oxidation of inorganic sulfur compounds to the reduction of nitrate, nitrite and other oxidized nitrogen compounds to molecular nitrogen (Kelly and Wood, 2000).

III. 1.1 Respiration and metabolism of *Thiobacillus denitrificans*

Thiobacillus denitrificans was one of the first non filamentous bacteria described to be able to grow with inorganic sulfur compounds as sole energy source (Kelly and Wood, 2000; Kelly *et al.,* 2005). *T. denitrificans* has the metabolic ability to obtain energy from the oxidation of reduced inorganic sulfur compounds under either aerobic or denitrifying conditions. Despite many years of work on the biochemistry of inorganic sulfur compounds oxidation by *T. denitrificans*, the mechanisms and how the electron transport is coupling to the oxidation is still not well understood (Beller *et al.,* 2006). The availability of the complete genome sequence should enable elucidation of the sulfur-oxidation pathway(s) and lead to specifically focused biochemical investigations to resolve these knowledge gaps (Beller *et al.,* 2006). Beller *et al.* (2006) presented the complete genome of *T. denitrificans,* which has the following features:

The genome of *T. denitrificans* strain ATCC 25259 consists of a single circular chromosome 2,909,809 bp in length with an average G+C content of 66.1%. *T. denitrificans* encodes all the necessary

enzymatic machinery for aerobic respiration. The enzymes can provide the reducing equivalents needed for terminal respiration either with nitrate or oxygen. The presence of both aa3- and cbb3-type oxidases, in addition to the denitrification machinery, allows *T. denitrificans* to survive under a wide range of redox conditions; presumably, the aa3 oxidase operates under high oxygen tension, cbb3 oxidases operate under microaerophilic conditions, and the denitrification complex operates under anaerobic conditions (Pereira and Teixeira, 2004; Pitcher and Watmough, 2004). *T. denitrificans* has all necessary genes encoding the four essential enzymes that catalyze denitrification. Organic storage materials in *T. denitrificans* have not been reported, but the presence of genes encoding glycogen synthase, maltooligosyl trehalose synthase, and various glucan branching enzymes (e.g., an α-1,4-D-glucan branching enzyme) and glucano- and glycosyl-transferases suggests that the bacterium synthesizes a polyglucose storage product (Beller *et al.,* 2006). The gene for the key enzyme of the Embden-Meyerhof-Parnas (EMP) pathway necessary for gluconeogenesis (fructose 1,6-bisphosphatase) is present in the genome (Beller *et al.,* 2006). Genes encoding alcohol dehydrogenase and other short-chain alcohol dehydrogenases, lactate dehydrogenase and phosphoketolase, suggest that *T. denitrificans* might be able to produce ethanol and lactate (by homo- or hetero-fermentative metabolism) from endogenous glucose under anoxic conditions (Beller *et al.,* 2006). Genes for all the enzymes of the Krebs tricarboxylic acid cycle were also identified in the genome.

The genome of *T. denitrificans* also encodes two sodium:solute symporter family proteins that may be involved in acetate uptake. A functional transport system for acetate in *T. denitrificans* was indicated by the uptake of 14C-labeled acetate into bacteria growing chemolithotrophically (acetate provided 6 to 11% of the cell carbon of strain ATCC 25259) (Timer-Ten, 1975). Pan and Umbreit (1972) showed that *T. denitrificans* grew in presence of glucose, nevertheless, in that study, it was not possible to know if the organic carbon was assimilated or not. Sublette and Woolsey (1988) showed that the H_2S oxidation by *T. denitrificans* was not affected by the presence of glutaraldehyde, however, in that experiment it was also difficult to know if the organic compound was utilized as energy or carbon source. These results indicate that *T. denitrificans* is able to tolerate the presence of organic matter, although it would be interesting to know if the ATCC 25259 strain has the metabolic capacity to respire or assimilate organic matter.

Sulfide oxidation by this kind of microorganisms can precede both under aerobic and anoxic conditions. Nowadays, there are not reports about the metabolic pathway of sulfide oxidation under

denitrifying conditions; however, there is a hypothetical metabolic pathway of sulfide oxidation under oxic conditions proposed by Visser *et al*. (1997) and shown on Figure 2. It is proposed that sulfide is oxidized to sulfate via intermediary sulfur and sulfite. Electrons enter the respiratory chain at the level of cytochrome c and are coupled to oxygen via a cbb3-type oxidase (Visser *et al*., 1997).

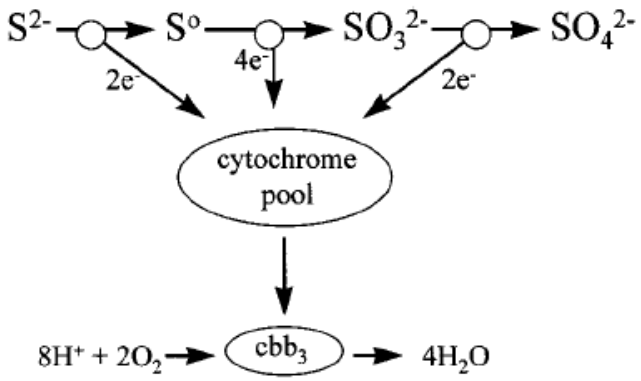

Figure 2 Schematic representation of the sulfide-metabolizing pathway in obligate autotrophic *Thiobacillus* (Visser *et al*., 1997).

Oxidation of sulfide by chemolithoautotrophic denitrifying bacteria can lead to the formation of elemental sulfur or sulfate, depending on the environmental conditions. Wang *et al.* (2005) observed that the elemental sulfur production was carried out on sulfide/nitrate molar ratio in the range of 1.66-2.5 and sulfide concentrations less than 300 mg/L. Reyes-Avila *et al.* (2004) carried out batch cultures in order to study the sulfide and acetate oxidation under nitrate limitation. These authors showed that the oxidation rate of sulfide to elemental sulfur was higher than the oxidation rate of acetate to carbon dioxide, and the elemental sulfur oxidation to sulfate was the slower reaction rate. These results show that the reaction rates are an important parameter to get the elemental sulfur production. Biotransformation of sulfide to elemental sulfur offers interesting opportunities for the removal of this compound, as the elemental sulfur has very low solubility in water and can be physically removed from effluents for reuse.

The stoichiometry of the sulfide oxidation under denitrifying conditions is shown in Eqs. (1) to (4). Comparison of Eqs. (1) and (2) shows that conversion to elemental sulfur coupled to complete denitrification consumes four times less nitrate as compared to complete oxidation to sulfate.

Comparison of Eqs. (1) and (3) illustrates a 2.5-fold larger consumption of nitrate in reactions involving incomplete denitrification to nitrite. All reactions are exergonics.

$S^{2-} + 1.6\ NO_3^- + 1.6\ H^+ \rightarrow SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O$ $\Delta G°' = -743.9$ kJ/ reaction (1)

$S^{2-} + 0.4\ NO_3^- + 2.4\ H^+ \rightarrow S^0 + 0.2\ N_2 + 1.2\ H_2O$ $\Delta G°' = -191.0$ kJ/ reaction (2)

$S^{2-} + 4\ NO_3^- \rightarrow SO_4^{2-} + 4\ NO_2^-$ $\Delta G°' = -501.4$ kJ/ reaction (3)

$S^{2-} + NO_3^- + 2\ H^+ \rightarrow S^0 + NO_2^- + H_2O$ $\Delta G°' = -130.4$ kJ/ reaction (4)

III. 1.2 Technological applications

The use of *T. denitrificans* or microbial denitrifying consortia is of interest for environmental technology because this biological process catalyzes the oxidation of sulfide and other reduced sulfur compounds in the absence of oxygen. The use of nitrate to control H_2S corrosion and odors in sewer systems has been known for many years and continues to be of commercial interest (Carpenter, 1932; Allen, 1949; Bentzen *et al.,* 1995). More recently, the addition of nitrate to sulfide-laden oil field brines was also shown to be an effective method to enhance the biological removal of sulfides and reduce problems associated with their toxicity, corrosivity and negative impact on reservoir permeability (Reinsel *et al.,* 1996; Jenneman *et al.,* 1999). Autotrophic denitrification has also been proposed for H_2S removal from biogas (Kleerebezem and Mendez, 2002). The concept has been investigated for industrial wastewater treatment (Gommers *et al.,* 1988; Reyes-Avila *et al.,* 2004; Sierra-Alvarez *et al.,* 2005). Microbial transformation of hydrogen sulfide to elemental sulfur offers interesting opportunities for the removal of this contaminant. Elemental sulfur is insoluble and can be physically removed from effluents for reuse. Alternatively, hydrogen sulfide can be oxidized to sulfate for discharge where sulfate is environmentally benign (e.g., marine environment). Numerous processes have been based on the use of S^0 for the autotrophic denitrification of drinking water (Van der Hoek *et al.,* 1992; Darbi *et al.,* 2003) or wastewater (Gommers *et al.,* 1988; Nugroho *et al.,* 2002; Am *et al.,* 2005) due to the high efficiency of nitrate consumption.

III. 2 Biodegradation of phenol under denitrifying conditions

The organotrophic denitrification is a biological process where the organic matter is the energy and carbon source, while the nitrate is the electron acceptor. There are diverse microorganisms involved

in the consumption and oxidation of aromatic compounds and such microorganisms use different strategies for the complete mineralization of the organic compounds (Philipp and Schink, 2000). Under oxic conditions, O_2 is used for destabilization and cleavage of aromatic compounds in oxygenase reactions (Dagley, 1971). In the absence of oxygen, the aromatic ring is destabilized by a reductive attack (Schink *et al.,* 1992; Heider and Fuchs, 1997). The most common and best studied pathway in anaerobic oxidation is the benzoyl- CoA pathway (Harwood *et al*., 1999). Considering the common occurrence of nitrate in many phenolic wastewaters, degradation of phenolics by denitrification seems to offer an attractive option for the overall treatment of phenolic wastewaters (Thomas *et al.,* 2002).

Practically, the parameters strongly influencing the success of phenolic compounds degradation include the mode of cultivation (batch, feed-batch or continuous), the presence or absence of other substrate than the contaminant tested, the type and size of the inoculum, and the kind of electron acceptor used (Watson, 1993; Razo-Flores *et al.,* 1996; Zaidi *et al.,* 1996; Hu *et al.,* 1998). Another important parameter is the adaptation phase, which has a crucial effect on the results of biodegradability studies, nevertheless, little is known about the phenomena involved in this process (Buitrón and Capdeville, 1995). Since the lipid membrane is the only barrier between the bacterial cytoplasm and the outside world, disruption of membrane function readily causes cell death (van Schie and Young, 2000). There are reports of bacteria that have developed mechanisms to resist and survive to high phenol concentration. One such mechanism is the isomerization of *cis*-unsaturated fatty acids to the *trans*-configuration, as it was seen by phenol-degrading *Pseudomonas putida* P8 (Heipieper *et al.,* 1992). The chains of *trans* fatty acids molecules can align closer together in a biological membrane than those in the *cis*-configuration, then a more rigid membrane is formed (van Schie and Young, 2000). Moreover, modifications in the lipid/protein ratio of the cell membrane in the presence of sublethal phenol concentrations have also been demonstrated (Keweloh *et al.,* 1990). These observations showed that the toxicity correlates well with the hydrophobicity of phenolic compounds (Liu *et al.,* 1982); it indicates that the cell membrane may be the main target of these antimicrobial agents (Heipieper *et al.,* 1991).

Only few phenol degrading microorganisms under denitrifying conditions have been isolated and characterized (Tschech and Fuchs, 1987; Paula *et al.,* 1998; Tong *et al.,* 1998; Shinoda *et al.,* 2000). Only two pure cultures of denitrifying bacteria that are able to use phenol as sole carbon and energy source are the strains K172 and S100 (Tschech and Fuchs, 1987). Strain K172 has been used in studies

of the biochemical pathways of both anaerobic phenol and toluene degradation (Schocher *et al.,* 1991; Lack and Fuchs, 1992, 1994). This microorganism was named *Thauera aromatica* and was classified as a new species of the genus *Thauera* (Anders *et al.,* 1995).

The pathway for phenol oxidation under denitrifying conditions has been elucidated in an *in vitro* system with cell free extracts of *T. aromatica* strain K-172. This organism was isolated from anaerobic sewage sludge, based on its ability to use phenol as its sole carbon and energy source and nitrate as the terminal electron acceptor (Tschech and Fusch, 1987). The initial steps in anaerobic phenol catabolism for the denitrifying strain K-172 have been studied (Figure 3) (Lack and Fuchs, 1992). The enzyme responsible for the carboxylation of phenol to 4-OH benzoate has been named phenol carboxylase. Purification of the phenol carboxylase from *T. aromatica* K-172 has been obstructed by the extreme oxygen sensitivity of the protein. It is known that only phenol induces its synthesis. Next three enzymes in the phenol pathway are also sensitive to oxygen but have been nonetheless purified. The synthesis of 4-OH-benzoyl-CoA-ligase (Biegert *et al.,* 1993), 4-OH-benzoyl-CoA-reductase (Brackmann and Fuchs, 1993), and benzoyl-CoA-reductase (Boll and Fuchs, 1995), are induced by aromatic substrates that are degraded via 4-hidroxybenzoate (4-OHBz).

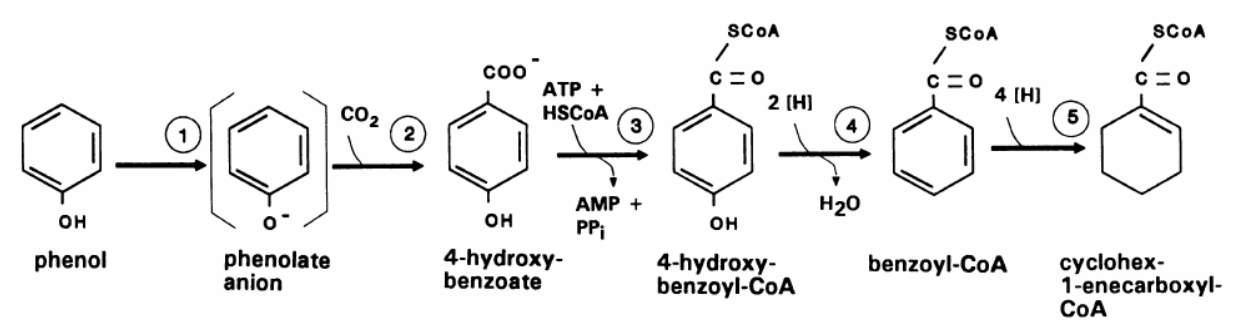

Figure 3 Intermediates and enzymes involved in the initial steps of anaerobic phenol metabolism in the denitrifying *Pseudomonas* strain K-172. (1) and (2), phenol carboxylase system; (3), 4-hydroxybenzoate-CoA ligase (AMP forming); (4), 4-hydroxybenzoyl-CoA reductase (dehydroxylating); (5), benzoyl-CoA reductase (aromatic ring reducing).

Benzoyl-CoA can be regarded as the central intermediate in the anaerobic oxidation of many aromatic compounds in *Thauera aromatica* K-172 and most likely also in other organisms capable of consuming aromatic compounds (Dangel *et al.,* 1991; Harwood and Gibson, 1997), as it can be seen in Figure 4.

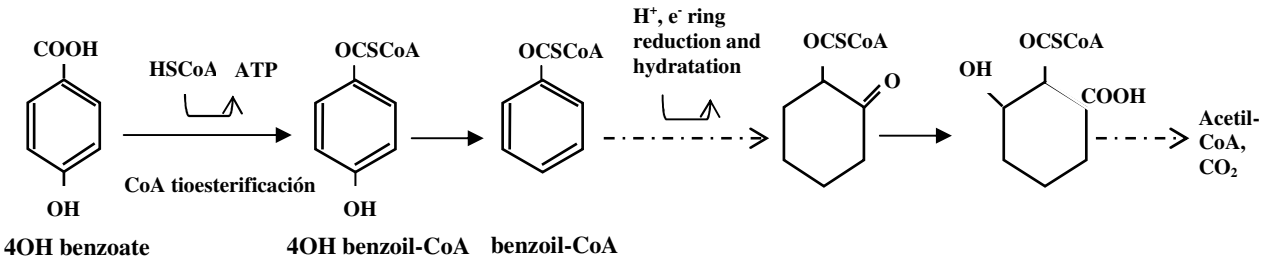

Figure 4 Oxidation of 4-hidroxybenzoate. This is the simplification of benzoate pathway reviewed by Harwood and Gibson (1997). The proposed reaction is based on data obtained from studies with *Rhodopseudomonas pallustris*, *Azoarcus evansii*, and *Thauera aromatica* K-172. The dashed arrows indicate reactions not known.

There are few studies about the phenol oxidation under denitrifying conditions. For example, Tschech and G. Fuchs (1987) showed in batch cultures that the phenol oxidation was coupled to denitrification, reducing nitrate to molecular nitrogen. The strains isolated from the granular sludge used were K-172 and S-100, which were identified as *Pseudomonas*. The oxidation of phenol under denitrifying conditions in batch cultures and continuous mode was also shown by Khoury *et al.* (1992). These authors did not detect organic residuals such as fatty acids or aromatic intermediates in the batch cultures. Nevertheless, in the continuous stirrer tank reactor the increase in the dilution rate (0.02-0.04 h^{-1}) affected the denitrification process, diminishing the phenol and nitrate consumption. Thomas *et al.* (2002) calculated the kinetic parameters for phenol oxidation under denitrifying conditions in batch mode with a mixed culture of *Alcaligenes faecalis* and *Enterobacter* species (8 mg/L of initial cell mass). The culture was capable of consuming high concentrations of phenol (up to 600 mg/L). The kinetic constants: maximum specific growth rate (μmax); inhibition constant, Ki and saturation constant, Ks were determined to be 0.206 h^{-1}, 113 and 15 mg phenol/L, respectively. p-Hydroxybenzoic acid was identified as an intermediate during phenol oxidation.

Puig-Grajales *et al.* (2003) showed the simultaneous elimination of phenol and 3,4-dimethylphenol (3,4 DMF) under denitrifying conditions using an UASB reactor at different COD/N-NO_3^- ratios. At a COD/N-NO_3^- ratio of 4.31 (240 mg/L of phenol and 38 mg/L of 3,4 DMF), the consumption efficiencies of phenol and nitrate were of 95%, while the consumption efficiency of 3,4 DMF was of 70%. Nitrate was completely consumed and reduced to molecular nitrogen. It was indicated that as nitrate was limited, it was not enough to oxidize completely the 3,4 DMF. Under these conditions, methane was detected (40% of biogas produced). These results showed that the phenolic compounds were

eliminated via denitrification and methanogenesis. At a COD/N-NO_3^- ratio of 2.57, the consumption efficiencies of phenol and 3,4 DMF were of 100%, while the consumption efficiency of nitrate was of 70%. The lower consumption efficiency of nitrate was due to the fact that nitrate was fed in excess. Methane was not detected under these last conditions. These results showed the competition between methanogenesis and denitrification for phenol and 3,4 DMF, however, denitrification was favored when nitrate was present in excess as electron acceptor. Klüber and Conrad (1998) also showed that nitrate concentrations of 30 mM still enabled a methane production of 25-40% of the one obtained before nitrate addition. These results indicate that only high concentration of nitrate can inhibit completely the methanogenesis.

In the last decades it has been demonstrated that it is possible to remove a second energy source such as sulfide or thiosulfate in presence of organic matter, coupled to the nitrate reduction. This biological process is called organo-lithotrophic denitrification.

III. 3 Organo-lithotrophic denitrification

Organo-lithotrophic denitrification is a biological process where organic and inorganic compounds are simultaneously used as electron donors coupled to the nitrate reduction. There is scarce information about this biological process. For instance, Gommers *et al.* (1988) observed the simultaneous oxidation of acetate and sulfide under denitrifying conditions in an ascendant fluidized bed reactor. During the steady state, the consumption efficiencies of acetate, sulfide and nitrate were of 100%. The end products formed were CO_2, SO_4^{2-}, N_2 and NO_2^-.

Kim and Son (2000) carried out batch cultures in order to study the effect of COD/N/S ratio on the denitrification process. The authors worked with a mixed culture of sulfate reducing bacteria and sulfur denitrifying bacteria, using acetate and thiosulfate as energy sources, and nitrate as electron acceptor. At a COD/N/S ratio of 0.8/1/3.3, the nitrate and thiosulfate were consumed in less than 6 hours, acetate was quickly consumed before 6 hours, and afterwards the acetate consumption was slow. At COD/N/S ratio of 3.3/1/3.3, the consumption rates for nitrate and thiosulfate were faster. However, the consumption of acetate was inhibited as it was indicated by the authors. Probably it was due to the reaction rates, since the consumed profiles showed that the specific rate of thiosulfate consumption was higher than the specific rate of acetate consumption.

Reyes-Avila *et al.* (2004) showed the simultaneous elimination of acetate and sulfide under denitrifying conditions using a continuous stirred tank reactor. The authors worked with a C/N ratio of

1.4 and an S/N ratio of 1.43, and a microbial consortium. Under steady state denitrification the consumption efficiencies of sulfide and nitrate were of 100%, while the consumption efficiency for acetate was of 65%. The end products were CO_2, N_2 and elemental sulfur. These authors also showed that, in presence of sulfide, acetate and nitrate, the slowest reaction was the elemental sulfur oxidation to sulfate in batch cultures. For this reason, the end product from sulfide oxidation was the elemental sulfur instead of sulfate.

There are several factors affecting the organo-lithotrophic denitrification, such as: stoichiometry of the reaction, initial substrate concentration, consumption specific rates, among others. These results showed that the denitrification process can be an alternative technology for simultaneous elimination of organic matter, sulfide and nitrate. Nevertheless, there is not information about simultaneous elimination of aromatic compounds such as phenol and sulfide under denitrifying conditions. Further studies about these factors will allow a better understanding of the denitrification in presence of two energy sources (aromatic and reduced sulfur compounds).

III. 4 Kinetic approach and evaluation of microbial respiratory process

The complexity of some wastewaters reveals that is not directly suited to optimize methods based on reaction kinetic principles. Nevertheless, by studying the microbial degradation kinetics of various compounds, the information can be gathered and used for understanding more complex systems in a step by step manner.

The balance for limiting substrate (usually it is the energy and carbon source for organo-heterotrophic microorganisms and the energy source for lithoautotrophic microorganisms) is shown in the following equation (Gómez, 2002):

$$S = S_x + S_p + S_R \qquad [5]$$

If equation (5) is derived with respect to the time, and the consumption efficiency of substrate is assumed to be of 100%, then:

$$-\frac{dS}{dt} = \frac{dS_x}{dt} + \frac{ds_p}{dt} \qquad [6]$$

It is experimentally known that:

$$\frac{ds}{dt} = q_s \cdot X \; ; \quad \frac{ds_x}{dt} = \frac{\mu X}{Y_{X/S}} ; \quad \frac{ds_p}{dt} = \frac{q_p X}{Y_{p/S}} \qquad [7]$$

Substituting equations (7) in equation (6), then:

$$qs = \frac{\mu}{Y\ X/S} + \frac{q_p}{Y\ P/S} \qquad [8]$$

The parameters of these equations are: initial concentration of substrate, S, substrate used for biomass growth, S_x, substrate used for product formation, S_p, residual substrate, S_R, specific rate of growth, μ, specific rate of product formation, q_p, specific rate of substrate consumption, q_S, and biomass concentration, X. Finally, $Y_{x/s}$ and $Y_{p/s}$ are the yields of biomass and product formed, respectively.

In batch culture through a dissimilative biological process the specific growth rate can be very small, having a very small biomass formation yield. Likewise, under dissimilative conditions the microbial double time is very long, so the microbial specific growth rate is very low. This indicates that in eq. (8) the term ($\mu/Y_{X/S}$) can be negligible, thus:

$$q_S = \frac{q_p}{Y\ P/S} \qquad [9]$$

In such cases, the energy formed will be dissipated in form of heat. The equation (9) suggests that the process depends mainly on the metabolic activity, rather on the microbial growth. The equation (9) also suggests that optimizing the microbial growth is not important.

An observation, known since the 1970's and still unsolved, is the often nonexistent transferability of kinetic data derived from batch cultures to continuous cultures and vice versa. This aspect is highlighted in the literature and the reliability of the kinetic parameters is questioned (Kovárová-Kovar and Egli, 1998). A change of the environmental conditions of the culture medium may lead to a change of the kinetic parameters. Consequently, to evaluate the performance of a microbial respiratory process, the following parameters must be taken in account: substrates consumption efficiencies, products or biomass yields, and substrate consumption or product formation specific rates.

IV Scope of the thesis

In this thesis, experimental studies concerning the biotransformation of various reduced sulfur compounds and phenol biodegradation under denitrifying conditions are discussed. The objective of this thesis was to evaluate the lithotrophic and organo-lithotrophic denitrification in batch and continuous cultures. Chapter 2 describes the effect of initial sulfide concentration on the kinetics and metabolism of simultaneous phenol and sulfide oxidation using a denitrifying sludge in batch cultures. Chapter 3 presents the simultaneous removal of phenol and sulfide under denitrifying conditions using an inverse fluidized bed reactor and the microbial ecology analysis of the biofilm. Chapter 4 presents the effect of initial nitrate and sulfide concentration, and different sulfur compounds (sulfide, thiosulfate and elemental sulfur) on a granular sludge activity produced under steady state denitrification in batch cultures. Finally, Chapter 5 describes the effect of elemental sulfur concentration on the rate of denitrification and products formed by a denitrifying granular sludge in batch cultures. In the same chapter, it is presented the rapid start-up of the "Sulfur-Limestone Autotrophic Denitrification" (SLAD) process in a bioreactor utilizing a granular sludge enrichment culture as inoculum.

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Chapter 2

Effect of initial sulfide concentration on metabolism of phenol oxidation under denitrifying conditions

ABSTRACT

The objective of this work was to evaluate the effect of initial sulfide concentration on the kinetics and metabolism of simultaneous phenol and sulfide oxidation using a denitrifying sludge in batch cultures. A complete oxidation of sulfide and phenol was coupled to nitrate reduction when nitrate was supplemented at stoichiometric concentrations. The sulfide and phenol oxidation was sequential, first sulfide was rapidly oxidized to elemental sulfur and afterwards to sulfate; phenol oxidation started once sulfate reached the maximum production. When initial sulfide concentration was increased from 20 to 26 and finally to 32 mg/L, the specific sulfide oxidation rates were not affected. Nonetheless, the denitrifying metabolism of phenol oxidation was affected as shown by both the production and accumulation of N_2O and a reduction of phenol and nitrate specific consumption rates, approximately 6.5 and 5.5 fold, respectively. Consequently, sulfide, phenol and possibly N_2O may have inhibited the denitrifying process.

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INTRODUCTION

Wastewaters from the chemical and petrochemical industry have an important environmental impact due to their high concentration of sulfide, ammonium and aromatic compounds (Olmos *et al.* 2004). These effluents represent a great challenge for treatment before discharge because of their chemical complexity. Sulfide is toxic, corrosive and a malodorous compound. Nitrogen compounds contribute mainly to eutrophication (Oenema and Roest, 1988). Phenolic compounds are the major organic constituents found in these effluents, and are toxic, carcinogenic, mutagenic and teratogenic (Autenrieth *et al.* 1991).

Biological nitrification–denitrification is the most common process for nitrogen removal from wastewaters. In the nitrification process, ammonium is aerobically oxidized to nitrite and then to nitrate; this compound can be reduced to molecular nitrogen by denitrifying microorganisms that use it as final electron acceptor. The ability to respire nitrate under anaerobic conditions is widespread among several genera either heterotrophic or autotrophic bacteria. Thus, in the denitrifying process nitrate reduction can be coupled to the oxidation of inorganic compounds such as sulfide (Sublette and Sylvester, 1987), and organic compounds, like phenol (Khoury *et al.* 1992; Puig-Grajales *et al*. 2003). Some denitrifying bacteria are chemolithoautotrophic and use reduced sulfur compounds such as elemental sulfur (S^0), sulfide (S^{2-}), thiosulfate ($S_2O_3^{2-}$), or sulfite (SO_3^{2-}) as electron donors (Schedel and Truper, 1980; Timmer-ten Hoor, 1981). The stoichiometry of the autotrophic denitrification using sulfide as electron donor is shown in Equations 1 and 2:

$$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O \qquad \Delta G^{\circ\prime} = -743.9 \text{ kJ/reaction} \qquad (1)$$

$$S^{2-} + 0.4NO_3^- + 2.4H^+ \rightarrow S^0 + 0.2N_2 + 1.2H_2O \qquad \Delta G^{\circ\prime} = -191.0 \text{kJ /reaction} \qquad (2)$$

Conversion of sulfide to elemental sulfur consumes four times less nitrate as compared to complete oxidation to sulfate.

Regarding organotrophic denitrifying bacteria, they are able to use a wide variety of organic compounds, such as phenol, 3,4-dimethylphenol, glucose, acetate, methanol, ethanol, etc. as electron donating substrates (van der Hoek *et al.,* 1987; Puig-Grajales *et al.,* 2003; Reyes-Avila *et al.,* 2004). The stoichiometric reaction for phenol oxidation under denitrifying conditions is shown in Equation (3).

$$C_6H_6O + 0.2H_2O + 5.6NO_3^- \rightarrow 6HCO_3^- + 0.4H^+ + 2.8N_2 \quad \Delta G^{\circ\prime} = -3071 \text{ kJ/ reaction} \qquad (3)$$

Several strains are known to be able of oxidizing phenol under denitrifying conditions, such as *Azoarcus sp.*, *Thauera aromatica* K172 and strain S100 (Tschech and Fuchs, 1987; Anders 1995; Shinoda *et al.* 2000). Another microorganism of great interest is the mixotrophic bacterium *Thiosphaera panthotropha* which is able to denitrify using reduced sulfur compounds, hydrogen or a wide range of organic compounds as electron donors (Kuenen *et al.* 1992; Chazal and Lens, 2000). This bacterium has been found and isolated from a fluidized bed reactor fed with sulfide and acetate as electron donors under denitrifying conditions (Gommers *et al.* 1998).

While there has been significant research on the biodegradation of phenolic compounds and reduced sulfur compounds under denitrifying conditions in a separate way, there is scarce information about the simultaneous oxidation of sulfide and phenol under denitrifying conditions. Only the simultaneous elimination of sulfide and acetate, a simple organic molecule, was studied in continuous and batch conditions (Gommers *et al*. 1988; Reyes-Avila *et al.* 2004). The main goal of this study was to evaluate the effect of initial sulfide concentration on the kinetics and metabolism of simultaneous phenol and sulfide oxidation under denitrifying conditions. For this purpose, batch cultures were conducted using anaerobic sludge as inoculum.

MATERIALS AND METHODS

Inoculum and Culture Medium Composition

The denitrifying sludge used for the batch experiments was cultivated in a 1.4 L upward-flow anaerobic sludge bed (UASB) reactor operated over a period of 2 months at a temperature of 32 ± 0.2°C with a hydraulic retention time of 1.5 d. The reactor was fed with an influent (pH 7.0) containing 0.049 g/L of sulfide, 0.742 g/L of nitrate and 0.193 g/L of phenol. The basal mineral medium was composed of (g/L): K_2HPO_4 (0.8), KH_2PO_4 (0.3), NH_4Cl (0.075), $MgCl_2$ (0.02), $NaHCO_3$ (0.17), and trace elements solution supplied at 2 mL/L. The trace element solution contained (g/L): EDTA (0.05), $CuSO_4 \cdot 5H_2O$ (0.015), $CaCl_2 \cdot 2H_2O$ (0.07), $MnCl_2$ (0.03), $(NH4)_6Mo_7O_{24} \cdot 4H_2O$ (0.015), $FeCl_3$ (0.015). The UASB was inoculated with 3 g volatile suspended solids (VSS) per liter of a denitrifying sludge, obtained from a laboratory-scale inverse fluidized bed reactor fed with synthetic water containing

sulfide, phenol and nitrate under steady state denitrification. Sludge samples from the UASB reactor were washed with saline solution to remove fine particles before use as inoculum in the batch tests.

Batch Culture studies

Batch cultures were conducted in 160 mL serum flasks containing 60 mL of mineral medium. The basal mineral medium was composed of (g/L): K_2HPO_4 (0.8), KH_2PO_4 (0.30), $MgCl_2$ (0.02), $NaHCO_3$ (0.0107). Trace elements solution with a composition similar to that used for the UASB was added (2 mL/L) to the medium. The serum bottles were spiked with variable concentrations of S^{2-} (20, 26 and 32 mg/L), 30 mg/L of NO_3^--N and 15 mg/L of phenol-C. The batch cultures were inoculated with 0.5 g VSS/L of denitrifying sludge. The headspace was flushed with helium for 10 min. Batch cultures were incubated at 30°C ± 2 on a shaker (200 rpm). Control batch cultures lacking NO_3^-, sulfide and/or phenol were also carried out. All batch cultures were performance in duplicate. To evaluate the overall performance of the denitrification process, phenol, 4-hydroxy-benzoate (a metabolite produced from phenol degradation), inorganic-C, NO_3^--N, NO_2^--N and N_2 concentrations were measured, and the specific rates were calculated from the kinetic data.

Analytical methods

To measure nitrate, nitrite, sulfate and thiosulfate, samples were centrifuged (9500g for 10 min) and filtered (0.45 µm) before being analyzed by capillary electrophoresis ion analyzer (Waters 4000). The reference electrolyte was sodium chromate 0.4 mM. A microcapillar of melted silica (60 cm long and 75 µm internal diameter) was used. The absorbance was measured in the ultraviolet region using a mercury lamp at 254 nm and 25ºC. The soluble inorganic carbon was measured using a TOC analyzer (Shimadzu TOC-5000A). N_2, N_2O and CO_2 were measured by gas chromatography (Varian 3350) with a thermal conductivity detector. Helium was used as mobile phase with a flow of 16 mL/min. The stainless steel column (Porapak Q mesh 100-80) was of 1.20 m long and 1/8" diameter. The injected sample volume was 50 µL. The temperatures were 50°C for the column and 100°C for detector and the injection port. The sulfide was measured by iodometric method (APHA, 1985). The biomass was measured as volatile suspended solids (VSS) per liter (APHA, 1985). Phenol and 4-hydroxy-benzoate were analyzed by HPLC (Perkin Elmer Series 200) with a column C-18. A mixture of acetonitrile/deionized water (60:40, v/v) was used as the solvent and the flow rate was maintained at 1.3 mL/min. Analysis was carried out at 225 nm.

RESULTS AND DISCUSSION

Continuous denitrifying UASB reactor

The UASB reactor was fed with constant loading rates of 112 ± 6.7 mg NO_3^--N/L·d, 98 ± 5.3 mg phenol-C/L·d, and 33 ± 2 mg S^{2-}/L·d. The molecular nitrogen production rate was 101 ± 5 mg/L·d. The denitrification process reached steady state as the molecular nitrogen production rate of the UASB reactor remained constant. During steady state the consumption efficiencies of phenol, sulfide and nitrate were close to 100%. Sulfide was not detected in the effluent and conversion of the consumed sulfide to SO_4^{2-} was 100%. Phenol was mainly mineralized and the conversion of the consumed phenol to HCO_3^- was 80%. The denitrifying sludge used in the present work for inoculating the batch cultures was usually produced under these conditions.

Control denitrifying batch cultures

Control batch cultures were carried out to determine the extent of contribution of the endogenous metabolism during the sulfide and phenol oxidation, as well as in the nitrate reduction. All control batch cultures had duration of 20 hours. In the biotic control with sulfide (29 mg/L) and lacking nitrate, only 5.5% of initial sulfide concentration was consumed. In the biotic control with phenol-C (13.28 mg/L) and lacking nitrate, the initial phenol concentration remained constant. Regarding the biotic control spiked with NO_3^--N (26 mg/L) and no electron donors, only 7% of initial nitrate concentration was consumed. These tests ensured that phenol and sulfide consumption were eliminated via denitrification process.

Denitrifying batch cultures

Firstly, denitrifying batch cultures were carried out with sulfide (Figure 1A) and phenol (Figure 1B) as the sole electron donors. In both cases, an immediate consumption of the different substrates (nitrate, sulfide and phenol) was observed without lag phase. The consumption efficiencies for all substrates were close to 100%. This was probably because the sludge came from a denitrifying UASB reactor fed with phenol and sulfide.

In the lithoautotrophic assays with 19 ± 0.53 mg S^{2-}/L and 13 ± 0.5 mg NO_3^--N/L (stoichiometric concentration), sulfide was quickly oxidized to SO_4^{2-}, reaching a sulfate yield of 0.90 ± 0.04 g SO_4^{2-}-S/g S^{2-} consumed after 70 hours (Figure 1A). It was observed that the color of the medium changed slightly from colorless to white. This is characteristic of the presence of elemental sulfur of colloidal

type (Krishnakumar, 1999; Beristain-Cardoso *et al*. 2006) and it suggests the transient formation of S^0 in the culture. Results showed that complete oxidation of sulfide to SO_4^{2-} was coupled to the denitrification process, obtaining a complete conversion of NO_3^- to N_2 with a molecular nitrogen yield of 1.03 ± 0.05 gN_2/ g NO_3^-- N consumed. These experimental results are in agreement with equation (1). Specific consumption rates were 72 mg NO_3^-- N/g VSS·d and 316 mg S^{2-}/g VSS·d.

With respect to the organotrophic assays with 13.34 ± 1.5 mg phenol-C/L and 16.6 ± 0.8 mg NO_3^--N/L (stoichiometric initial concentration), phenol was totally oxidized to CO_2, reaching a inorganic carbon yield of 0.9 ± 0.03 g /g phenol-C consumed (Figure 1B). The phenol oxidation was coupled to the denitrification process, obtaining a complete conversion of NO_3^- to N_2 with a molecular nitrogen yield close to 1. These experimental results are in agreement with equation (3). Specific consumption rates were 68 mg NO_3^-- N/g VSS·d and 61 mg phenol-C/g VSS·d. These results showed the metabolic capacity of the consortium to use sulfide and phenol separately as electron donors for denitrification. Nonetheless, it is interesting to note that the specific consumption rate for sulfide was 5 times higher than the specific consumption rate for phenol. This could be due to different factors, such as substrates transport velocities, composition of the microbial consortium, and biomass affinity for substrates. The slow oxidation of sulfide to sulfate was probably associated to the transient accumulation of elemental sulfur as intermediate. As previously demonstrated by Reyes-Avila *et al*. (2004), the oxidation of elemental sulfur to sulfate seemed to be the limiting step in the sulfide oxidation to sulfate.

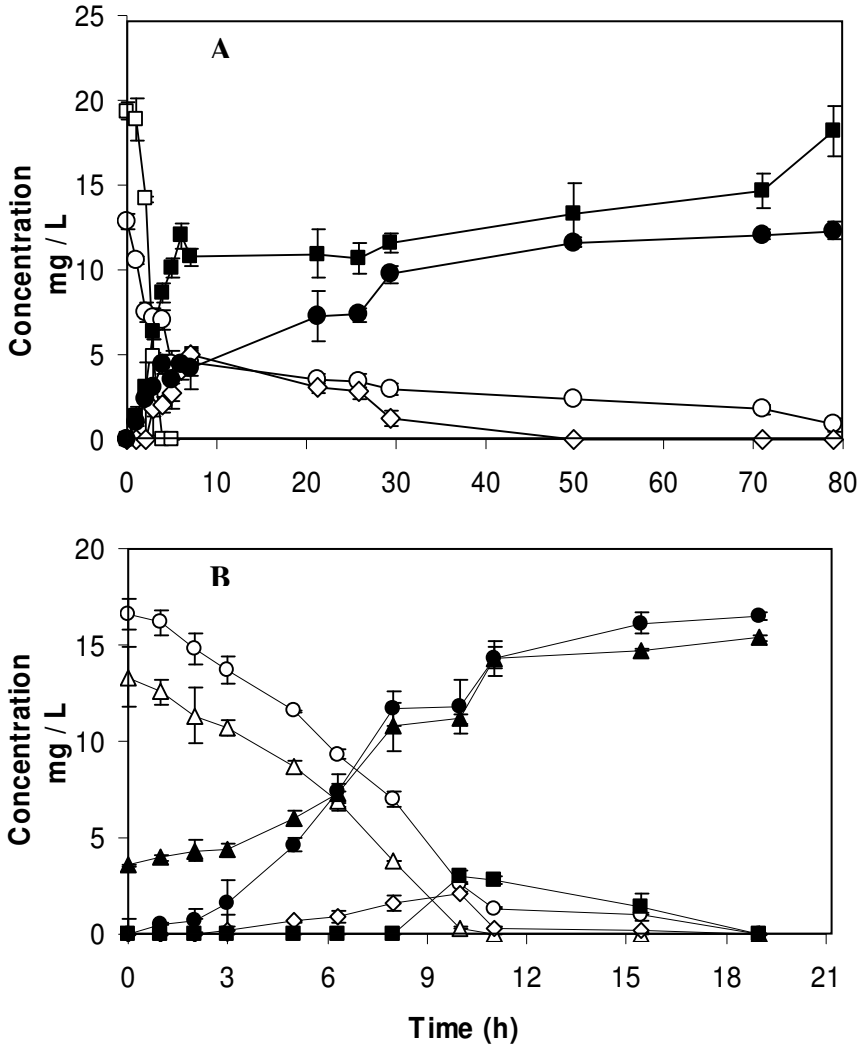

Figure 1 Kinetic profiles of nitrogen, carbon and sulfur compounds in denitrifying cultures: **A**) S^{2-} as sole electron donor, **B**) phenol as sole electron donor. (○) NO_3^--N; (◊) NO_2^--N; (•) N_2; (□) S^{2-}; (■) SO_4^{2-} -S; (Δ) phenol-C; (▲) inorganic-C; (■) 4hydroxy-benzoate.

Effect of initial sulfide concentration on denitrifying process with phenol

Batch cultures under mixotrophic conditions (phenol plus sulfide) were carried out with different initial concentration of sulfide (20, 26 and 32 mg/L) while initial concentrations for phenol (15 mg phenol-C/L) and nitrate (30 mg NO_3^--N/L) were maintained constant. According to equations (1) and (3), the batch culture conditions with 20 mg/L of S^{2-} presented the stoichiometric concentration necessary for complete oxidation of both phenol and sulfide. In the batch cultures with 26 and 32 mg/L of S^{2-}, there was an excess of sulfide of 30 and 60% as electron donor, respectively.

Theoretically, nitrate concentration was not enough to have a complete oxidation of both sulfide and phenol.

At 20 mg/L of sulfide, consumption efficiencies of sulfide, phenol and nitrate were close to 100% and inorganic carbon, sulfate and molecular nitrogen yields were 0.93 ± 0.04 g /g phenol-C consumed, 1.14 ± 0.11 g SO_4^{2-}-S/g S^{2-} consumed and 0.95 ± 0.06 g N_2/ g NO_3^-- N consumed, respectively (Figure 2). Sulfide was rapidly consumed at a specific rate of 370 mg/g VSS·d. Once sulfate yield reached a value of 1.14 ± 0.11 g SO_4^{2-}-S/g S^{2-} consumed, phenol consumption started after a lag phase of 5 hours and at specific rate of 104 mg/g VSS-d. Probably these specific rates increased due to a greater initial concentration of nitrate with respect to the lithoautotrophic and organotrophic assay. Beristain-Cardoso *et al*. (2006) showed that the specific consumption rates of nitrate and sulfide increased with the increment of initial nitrate concentration. When both sulfide and phenol were available at stoichiometric initial concentrations in the culture medium, the denitrifying sludge was able to achieve the total oxidation of S^{2-} to SO_4^{2-} and phenol to CO_2. These oxidation processes were coupled to the reduction of nitrate to N_2. The results presented in this work showed the metabolic capacity of the denitrifying sludge to use both sulfide and phenol as simultaneous electron donors.

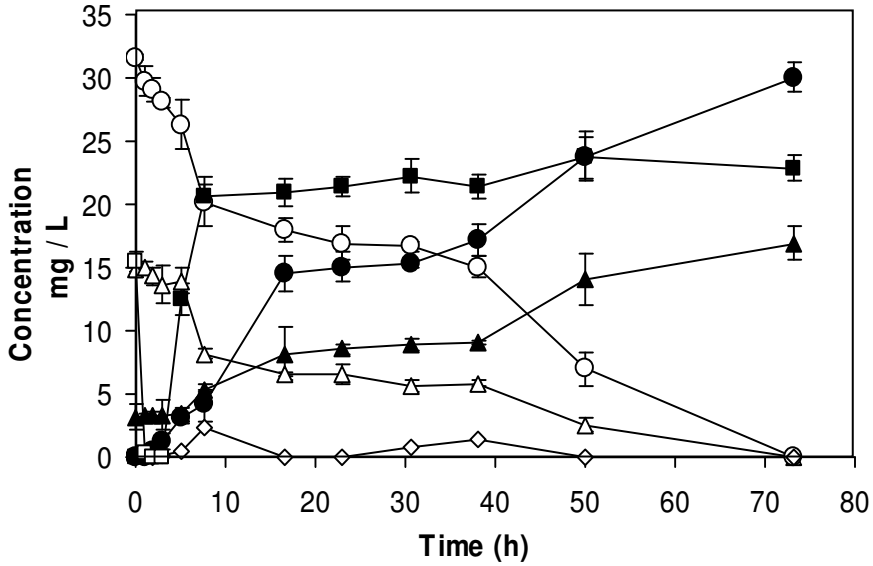

Figure 2 Kinetic profiles of nitrogen, sulfur and carbon compounds in denitrifying cultures with sulfide (20 mg/L) and phenol as electron donors. (○) NO_3^--N; (◊) NO_2^--N; (•) N_2; (□) S^{2-}; (■) SO_4^{2-}-S; (Δ) phenol-C; (▲) inorganic-C.

The results obtained at sulfide concentration of 26 mg/L are presented on Figure 3. The consumption efficiencies of sulfide, phenol and nitrate were 100, 60 and 75%, respectively. The sulfate, inorganic carbon and molecular nitrogen yields were 0.79 ± 0.04 g SO_4^{2-}-S/g S^{2-} consumed, 0.97 ± 0.03 g/g phenol-C consumed and 0.74 ± 0.05 g N_2/ g NO_3^-- N consumed, respectively. Under this culture conditions, N_2O was detected during phenol oxidation, indicating that the activity of the enzyme N_2O reductase from the denitrifying metabolic pathway was possibly affected. At the end of the experiment, there was enough reducing source (phenol) to eliminate the residual NO_3^-, however, the accumulation of N_2O in the culture might have inhibited the denitrifying process, leading to a decrease in the consumption efficiencies for nitrate and phenol. It must be noted that the lag phase of phenol consumption increased from 5 h in the previous experiment to 10 h at 26 mg/L of sulfide.

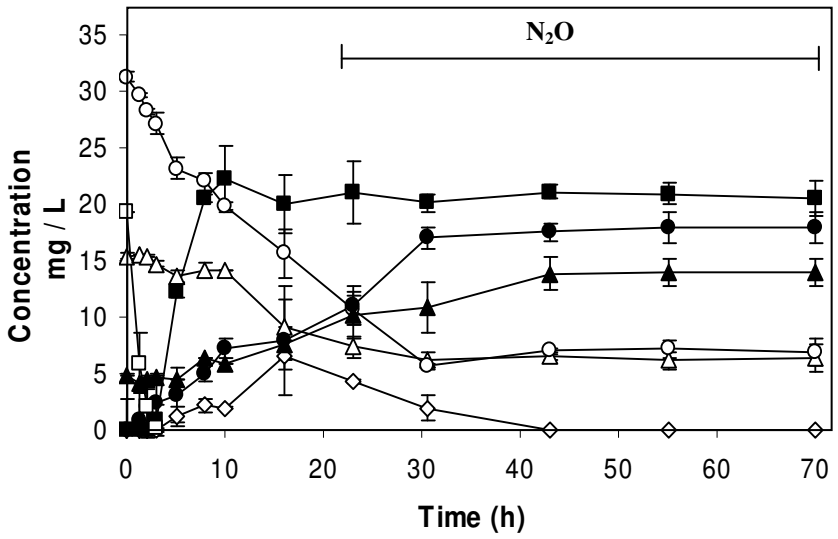

Figure 3 Kinetic profiles of nitrogen, sulfur and carbon compounds in denitrifying cultures with sulfide (26 mg/L) and phenol as electron donors. (○) NO_3^--N; (◊) NO_2^--N; (•) N_2; (□) S^{2-}; (■) SO_4^{2-}-S; (Δ) phenol-C; (▲) inorganic-C.

The results obtained at sulfide concentration of 32 mg/L are presented on Figure 4. The consumption efficiencies of sulfide, phenol and nitrate were 100, 84 and 87%, respectively. The sulfate, inorganic carbon and molecular nitrogen yields were 0.80 ± 0.02 g SO_4^{2-}-S/g S^{2-}, 0.83 ± 0.02 g/g phenol-C consumed and 0.90 ± 0.05 g N_2/ g NO_3^-- N consumed, respectively. Phenol consumption started after a lag phase of 14 h at a specific oxidation rate of 15 mg/g VSS-d, while the specific sulfide oxidation rate did not significantly change. In this case, N_2O was also detected during sulfide oxidation process.

Sorensen *et al*. (1980) observed in batch culture with denitrifying *Pseudomonas fluorescens* that the enzyme N_2O reductase was inhibited at a concentration of 10 mg H_2S/L, obtaining N_2O as end product instead of N_2. The fact that, at a higher concentration of sulfide (32 mg/L), N_2O accumulated in the culture could be due to a possible inhibitory effect of sulfide on the enzyme N_2O reductase.

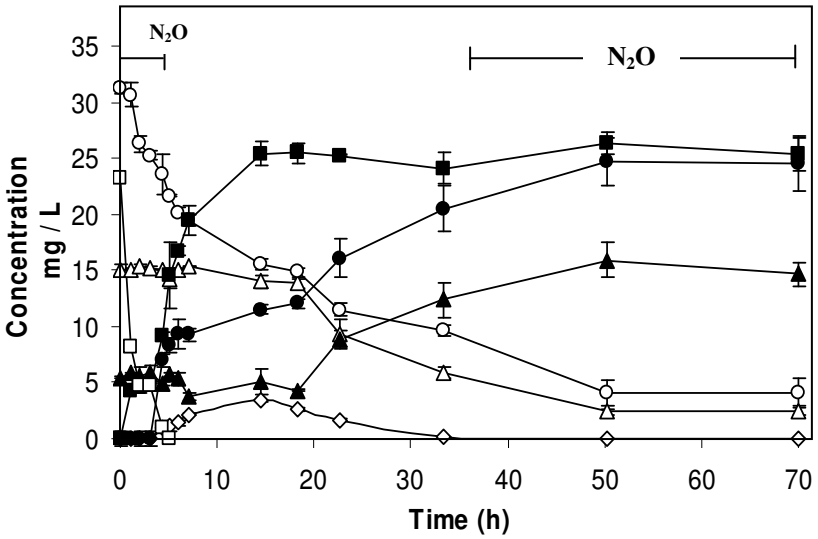

Figure 4 Kinetic profiles of nitrogen, sulfur and carbon compounds in denitrifying cultures with sulfide (32 mg/L) and phenol as electron donors.. (○) NO_3^--N; (◊) NO_2^--N; (•) N_2; (□) S^{2-}; (■) SO_4^{2-}-S; (Δ) phenol-C; (▲) inorganic-C.

Results showed that when sulfide and phenol were present at stoichiometric initial concentrations, oxidation processes did not occur simultaneously. Sulfide was first oxidized to sulfate and phenol was successively consumed. The exposition time of phenol on denitrifying sludge increased due to the lag phase of phenol. The lag phase of phenol oxidation increased with the initial concentration of sulfide in the medium. This exposition time of phenol could affect the denitrifying sludge and this caused a decrease in the phenol and nitrate consumption rates (Table 1), accumulating N_2O. The N_2O formation could contribute to the inhibition or toxicity, which originated that the denitrification process completely stoped.

At higher concentration of sulfide, accumulation of elemental sulfur as transient intermediate from the sulfide oxidation increased, being the oxidation of S^0 to sulfate the limiting step in sulfide oxidation process (Table 1). This could be associated to the increase in the lag phase for phenol

oxidation; however, further work is required in this direction to better understand the process involved.

As shown in Table 1, when the initial concentration of sulfide increased, the specific phenol consumption rate values decreased significantly while specific sulfide oxidation rates did not change. Reyes-Avila et al. (2004) studied the simultaneous oxidation of sulfide and acetate under denitrifying conditions in batch and continuous cultures. The denitrifying biomass from the continuous stirred sulfide oxidizing reactor was used for batch cultures, with an initial biomass concentration of 1.45 g VSS/L. These authors suggested a sulfide and acetate oxidation pathway with the following sequence for reaction rates (q): $q_S^{2-} > q_{Acetate} > q_S^0$. In the present work, the order of reactions rates was found to be: $q_S^{2-} > q_S^0 = q_{SO4}^{2-} > q_{Phenol}$ (Table 1). In the same way, Reyes-Avila *et al.* (2004) found that, with acetate, the end products were S^0, CO_2 and N_2, while, in the present work with phenol, the end products were SO_4^{2-}, CO_2 and N_2. These results indicate that the chemical structure of the organic compounds could modify the outcome of the denitrification products.

Table 1 Consumption specific rates and sulfate formation rates at different initial concentration of sulfide

Initial sulfide concentration (mg/L)	Phenol oxidation (mg phenol-C / g VSS d)	Sulfide oxidation (mg S^{2-}/ g VSS d)	Sulfate formation (mg SO_4^{2-}-S/ g VSS d)
20	104 ± 11	370 ± 35	209 ± 21
26	17.6 ± 3	297 ± 23	122 ± 25
32	16.0 ± 5	283 ± 34	50 ± 11

CONCLUSIONS

A complete oxidation of sulfide and phenol was coupled to the nitrate reduction when nitrate was supplemented at stoichiometric concentrations. The sulfide and phenol oxidation was sequential, first sulfide was oxidized to sulfate, and afterwards phenol was successively consumed. When initial sulfide concentration was increased from 20 to 26 and finally to 32 mg/L the specific sulfide oxidation rates were not significantly affected, while denitrifying metabolism was affected as shown by both N_2O accumulation and a reduction of the specific phenol and nitrate consumption rate values. The combined effect of sulfide plus phenol possibly inhibited the denitrifying process since efficiencies of phenol and nitrate consumption also decreased. These results suggest that a denitrifying system may have promising applications for complete removal of phenol and sulfide from wastewaters.

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CHAPTER 3

Simultaneous phenol and sulfide removal by denitrification using an inverse fluidized bed reactor and its microbial community analysis

ABSTRACT

A microbial consortium attached onto a polyethylene support was used to evaluate the simultaneous removal of sulfide and phenol under denitrifying conditions. The phenol, sulfide and nitrate loading rates applied to an inverse fluidized bed reactor were up to 168 mg phenol-C/l d, 37 mg S^{2-}/l d and 168 mg NO_3^--N/l d, respectively. Under steady state operation the consumption efficiencies of phenol sulfide and nitrate were 100%. The N_2 yield (g N_2/g NO_3^--N consumed) was 0.89. The phenol was mineralized resulting in a yield of 0.82 g inorganic-C/g phenol-C consumed and sulfide was completely oxidized to sulfate with a yield of 0.99 g SO_4^{2-}-S/g S^{2-} consumed. The microbial analysis of 16S rDNA on the denitrifying biofilm showed the presence of *Thiobacillus denitrificans*, *T. sajanensis* and *Thiobacillus sp.* This is the first work reporting the simultaneous removal of sulfide and phenol using a denitrifying biofilm reactor.

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INTRODUCTION

Nowadays there is a growing interest to develop technologies for the removal of complex mixtures of organic and inorganic compounds from industrial wastewaters such as the chemical and petrochemical industry. These wastewaters have an important environmental impact due to their high concentration of sulfide, ammonium and aromatic compounds (Olmos *et al.,* 2004). Sulfide is toxic, corrosive and a malodorous compound. There are several studies which show that sulfide is inhibitory for many microorganisms. This inhibitory effect is presumed to be caused by unionized hydrogen sulfide, because only neutral molecules permeate well the cell membrane (Reis et al., 1992; Oude Elferink et al., 1994). Another possibility is that sulfide can combine with the iron of ferredoxin and cytochrome in the cell, stopping the electron transport system (Hao et al. 1996).

The phenolic compounds are toxic, carcinogenic, mutagenic and teratogenic when they are found in eutrophying concentrations (Autenrieth et al., 1991). The phenolics compounds have an important effect on bacterial membrane; however, there are reports of bacteria that have developed mechanisms to resist to high phenol concentration. One such mechanism of resistance is the isomerization of cis-unsaturated fatty acids to the trans-configuration, as it was seen by phenol-degrading *Pseudomonas putida* P8 (Heipieper et al., 1992).

On the other hand, wastewater streams containing nitrogenous compounds may cause serious environmental problems if these compounds are not properly removed before discharge into the receiving water bodies. A high nitrogen concentration in the receiving waters can lead to eutrophication, i.e. algal outbreaks and/or fish death in rivers, lakes, and coastal areas (Oenema and Roest, 1988; Metcalf and Eddy, 1991; Mussati et al., 2002).

Sulfide, phenol and nitrogen compounds can be eliminated by physicochemical or biological methods. The physicochemical methods are expensive because of the consumption energy and other chemical substances, generating undesirable and toxic residual products that need further treatment. Anaerobic biological processes have been considered as alternatives to remove aromatic compounds (Field *et al.,* 1995). Phenol biodegradation has been reported under sulfate reduction (Smolensky and Suflita, 1987), methanogenic (Razo-Flores *et al.,* 1996), humus reducing (Cervantes *et al.,* 2000) and denitrifying (Thomas *et al.,* 2002) conditions. The stoichiometric reaction for phenol oxidation under denitrifying conditions is shown in Equation (1), indicating that the C/N stoichiometric ratio is 0.92.

$$C_6H_6O + 0.2H_2O + 5.6NO_3^- \rightarrow 6HCO_3^- + 0.4H^+ + 2.8N_2 \quad \Delta G^{\circ\prime} = -3071 \text{ kJ/ reaction} \qquad (1)$$

Some denitrifying bacteria are chemolithoautotrophic and use reduced sulfur compounds such as elemental sulfur (S^0), sulfide (S^{2-}), thiosulfate ($S_2O_3^{2-}$), or sulfite (SO_3^{2-}) as electron donors (Schedel and Truper, 1980; Timmer-ten Hoor, 1981; Beristain-Cardoso et al., 2006). The stoichiometry of the complete sulfide oxidation under denitrifying conditions is shown in Equation (2), indicating that the stoichiometric S/N ratio is 1.43.

$$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O \qquad \Delta G^{\circ\prime} = -743.9 \text{ kJ/reaction} \qquad (2)$$

Comparing the organotrophic and lithotrophic ΔGº′ values (equations 1 and 2) show that the first one is more spontaneous. However, Reyes-Avila et al., (2004) indicated that the lithoautrophic process is faster using acetate as electron donor.

Sulfide can be removed via anaerobic processes by chemolithoautotrophic microorganisms such as *Thiobacillus denitrificans*, which has been extensively studied under anoxic or aerobic conditions (Sublette and Silvester, 1987; Buisman et al., 1993; Wang et al., 2005). There are evidences suggesting that during the denitrification process nitrate reduction can be coupled to the simultaneous oxidation of sulfide and simple organic molecules such as acetate in continuous and batch mode (Gommers et al., 1988; Reyes-Avila et al., 2004). The simultaneous removal of sulfide and phenol using a denitrifying biofilm has not been reported. Nonetheless, it has been demonstrated that biofilm structures allow attached microorganism to tolerate high concentrations of toxicants (i.e., sulfide) without any apparent toxic effect (Celis et al., 2004, 2007). Therefore, the objective of this study was to evaluate the simultaneous removal of sulfide and phenol under well defined stoichiometric denitrifying conditions using a biofilm reactor such as the inverse fluidized bed reactor (IFBR).

MATERIALS AND METHODS

Inverse fluidized bed reactor and culture medium composition

The IFBR used consisted of a glass column of nominal volume of 2 L (1.14 m of height and 4.8 cm of internal diameter) and a working volume of 1.7 L. The experimental set up of the IFBR is shown in

Figure 1. The biofilm carrier particle was low density polyethylene (267 Kg/m^3) of 0.4 mm mean diameter, occupying 20% of the working volume. The mineral medium (pH 7.0) was prepared as follows (g/L): K_2HPO_4 (0.80); KH_2PO_4 (0.3), NH_4Cl (0.15); $MgCl_2$ (0.02); $NaHCO_3$ (0.16), and trace elements solution supplied at 2 ml/L. The trace element solution contained (g/L): EDTA (0.05), $CuSO_4 \cdot 5H_2O$ (0.015), $CaCl_2 \cdot 2H_2O$. The sulfide and phenol were the electron donors, while nitrate was the electron acceptor. The working temperature was 30°C ± 1. The down-flow liquid velocity was 4 m/h and the bed expansion achieved was between 50 and 60% of the working volume. Molecular nitrogen produced was measured in a calibrated column by liquid displacement.

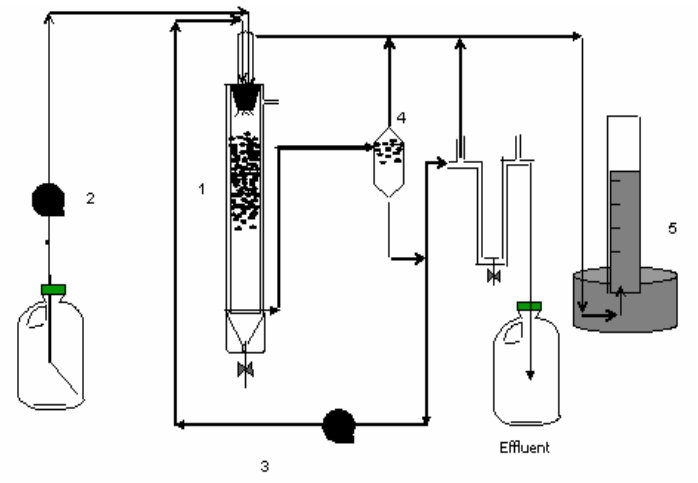

Figure 1 Scheme of the IFBR and devices: 1) Bioreactor, 2) influent pump 3) Recirculation pump 4) sludge trap and 5) biogas collection

Inoculum, biomass immobilization and continuous operation

The IFBR was operated in batch mode for 5 days to allow the microbial colonization of the plastic support using phenol (180 mg/L) as carbon and energy source, and nitrate (490 mg/L) as electron acceptor. The IFBR was inoculated with a denitrifying sludge obtained from a lab scale continuous stirred tank reactor that treated nitrate and acetate; under steady state conditions the consumption efficiencies of nitrate and acetate were 100%. The initial concentration of volatile suspend solids (VSS) in the IFBR was of 0.9 g/l. The liquid of the reactor was replaced with freshly prepared medium every 24 hours (for 5 cycles). Only the immobilized volatile solids (IVS) at the end of the batch operation were measured, being of 1.2 kg IVS m^{-3} of dry support.

After the polyethylene particles were colonized, the IFBR was operated in a continuous mode under organotrophic conditions fed with phenol and nitrate. The IFBR was operated with two hydraulic retention times (HRT); 2 and 0.9 days. The experimental C/N ratio was 1.05, with a 13% carbon excess according to eq. 1. Once the IFBR reached the steady state under organotrophic conditions at HRT of 0.9 d, it was fed with sulfide as a second electron donor, at a constant loading rate of 37 mg S^{2-}/l d. The NO_3^--N fed was enough to completely oxidize the sulfide to sulfate and also to oxidize approximately 87% of phenol to CO_2. The denitrification was evaluated through the consumption efficiencies of substrate and the product yields: denitrifying yield (g N_2/g NO_3^- -N consumed), bicarbonate yield (g HCO_3^--C/g phenol-C consumed) and sulfate yield (g SO_4^{2-}-S/g S^{2-} consumed).

Chemical analysis

Nitrate, nitrite, and sulfate were measured in a capillary electrophoresis ion analyzer (Waters 4000). The reference electrolyte was sodium chromate 0.4 mM. A microcapillar of melted silica (60 cm long and 75 µm internal diameter) was used. The absorbance was measured in the ultraviolet region using a mercury lamp at 254 nm and 25ºC. The total inorganic and organic carbon (TOC) was measured using a TOC analyzer (Shimadzu TOC-5000A). At the experimental pH value of 7.0, the carbonate speciation is as follow: $H_2CO_{3\ (aq)}$; 17.9%, $HCO_{3\ (aq)}^-$; 82.1% and $CO_{3\ (aq)}^{2-}$; 0.038% (Warner and Morgan, 1995). Thus we assumed that the soluble total inorganic carbon detected for the TOC analyzer is bicarbonate. N_2 and CO_2 were measured by gas chromatography (Varian 3350) with a thermal conductivity detector. Helium was used as mobile phase with a flow of 16 ml/min. The stainless steel column (Porapak Q mesh 100-80) was of 1.20 m long and 1/8" diameter. The injected sample volume was 50 µL. The temperatures were 50°C for the column and 100°C for both the detector and the injection port. The sulfide was measured by iodometric method (APHA, 1985). The free biomass, and attached biomass (after detachment for sonication) were measured as volatile solids (VS) (APHA, 1985). Phenol was analyzed by HPLC with a column C-18. A mixture of acetonitrile/deionized water (60:40, v/v) was used as the solvent and the flow rate was maintained at 1.3 ml/min (Perkin Elmer Series 200, Shelton USA). The absorbance was measured at 225 nm. In all cases the variation coefficient was close of 8%.

16S rDNA analysis

At the end of the IFBR experiment the genomic DNA in the biofilm was extracted using a method adapted from Wisotzkey et al. (1990). The extracted DNA was amplified by polymerase chain reaction (PCR) using an automated thermal cycler (Techne Model Touchgen Gradient) as follows: an initial denaturation at 94°C for 3 min; 35 cycles of: denaturation (30 sec at 94°C), annealing (30 sec at 65°C) and extension (1 min at 72°C); a final extension at 72°C for 7 min and then stored at 4°C. All PCR amplifications were conducted in 25 µl of a mixture reaction containing Taq Buffer pH 8.5, 5 nmol of each deoxynucleotide thriphosphates (dNTPs), 50 nmol $MgCl_2$, 50 pmol of each primer and Go Taq Flexi DNA Polymerase 5 U/µl (Promega). The 16S rDNA from the mixed bacterial DNA was amplified using primers for the kingdoms Bacteria (533F) and Archeobacteria (1381 R). The PCR products were evaluated on a 1% (wt/vol) agarose gel electrophoresis. The DNA on the gel was visualized under UV light after stained with 0.5 µg ethidium bromide/ml. PCR products were purified by using a QIAquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The amplified bacterial 16S rDNA fragments purified were ligated into the pGEM-easy vector cloning system (Promega) and transformed into competent cells (*Escherichia coli* TOP 10). The total transformed colonies were selected for inoculation in a 3.0 ml LB medium containing 100 mg/L carbeneciline. After 12 h incubation at 37°C, the plasmids were recovered using the method of Birboim and Doly, (1979). Less than 10 clones were selected. The DNA was purified by using a QIAquick Spin PCR purification kit and quantified by Fluorometer Tecan model Genios. The purified PCR products were sequenced using fluorescent dye labeled dideoxynucleotides and an ABI Prism Model 377 DNA sequencer (Applied Biosystems, Foster City, CA). The obtained sequences were compared with the existing 16S rDNA sequences in the nucleotide database of the National Center for Biotechnology Information (NCBI) using BLAST 2.1 (Altschul et al., 1997).

RESULTS AND DISCUSSION

Organotrophic denitrification in continuous mode at different loading rates

The attached biomass was measured as kilograms of immobilized volatile solids per cubic meter of dry support (kg IVS/m^3). The attached biomass in batch mode was 1.2 Kg IVS/m^3.

At HRT of 1.9 days, the molecular nitrogen production rate was of 64 ± 5 mg/l·d. As the variation was less than 10% the denitrification was in steady state. Under these culture conditions the consumption efficiencies of phenol and nitrate were close to 100%. Phenol was mineralized, reaching a bicarbonate

yield of 0.84 g HCO_3^--C/g phenol-C consumed. The phenol oxidation was coupled to the denitrification process, obtaining a complete conversion of NO_3^- to N_2, as the molecular nitrogen yield was close to 1 g N_2/g NO_3^--N. The biological oxidization-reduction process was in agreement to equation 1. The mass balance for nitrogen and carbon compounds was as follows:

65.90 mg $N_{Influent}$/l·d = [(64 mg N_2/l·d) + (1.71 mg suspended biomass-N/l·d)] $_{Effluent}$ = 65.71 mg N $_{Effluent}$ /l·d

70.71 mg $C_{Influent}$/l·d = [(57.73 mg HCO_3^--C/l·d) + (1.5 mg $CO_{2\ (gas)}$-C/l·d) + (8.5 mg suspended biomass-C/l·d)] $_{Effluent}$ = 67.7 mg C $_{Effluent}$ /l·d

The recovery percentages of nitrogen and carbon products were 99 and 95% from the substrates consumed, respectively. The unrecovered carbon was possibly used for the production of biomass.

The loading rates for nitrate and phenol were increased stoichiometrically at 87 mg N-NO_3^- /l·d and 90 mg C-phenol /l·d. Under steady state operation the consumption efficiencies of phenol and nitrate, as well as the yield values did not significantly change. Thus, the increase of the loading rates did not affect the denitrifying process.

Afterwards, the loading rates for phenol and nitrate were increased by diminishing the HRT from 1.9 to 0.9 d. During steady state operation the consumption efficiencies of phenol and nitrate were close to 100%. The molecular nitrogen production rate was of 208 ± 5 mg/l·d, with a yield of 0.99 ± 0.02 g N_2/g NO_3^--N consumed. The denitrification was stoichiometrically coupled to phenol oxidation, obtaining a bicarbonate yield of 0.79 ± 0.04 g HCO_3^--C/g phenol-C consumed. The mass balance for nitrogen and carbon compounds was as follows:

211 mg $N_{Influent}$/l·d = [(208 mg N_2/l·d) + (4 mg suspended biomass-N/l·d)] $_{Effluent}$ = 212 mg $N_{Effluent}$ /l·d

188.5 mg $C_{Influent}$/l·d = [(137 mg HCO_3^- -C/l·d) + (13 mg $CO_{2\ (gas)}$ -C/l·d) + (19 mg suspended biomass-C/l·d)]$_{Effluent}$ = 169 mg $C_{Effluent}$ /l·d

The recovery percentages for nitrogen and carbon products were 100 and 89% of substrates consumed, respectively. The HRT diminution did not affect the respiratory process of denitrification. Puig-Grajales et al. (2003) showed that under denitrifying conditions the simultaneous elimination of

phenol (240 mg/l) and 3,4 dimetylphenol (38 mg/l) is possible. The consumption efficiencies of phenol and 3, 4 dimetylphenol were 95 and 70%, respectively. However, methane was detected indicating that the phenolic compounds were biodegraded by denitrification and methanogenesis. Under our experimental conditions no methane was detected.

Simultaneous sulfide and phenol removal by denitrification

After 75 days of operation under organothrophic conditions in continuous mode, sulfide was fed to the IFBR at a constant loading rate of 37 ± 5 mg/l d for 33 additional days (Figure 2). The molecular nitrogen production rate was 150 ± 9.5 mg/l·d. The consumption efficiencies of phenol, sulfide and nitrate were close to 100%. Phenol was mainly mineralized, reaching a bicarbonate yield of 0.82 ± 0.04 g HCO_3^--C/g phenol-C consumed. The biomass determined in the reactor effluent was 23 ± 4 mg biomass-C/l·d. Sulfide was completely oxidized to sulfate, reaching a sulfate yield of 0.99 ± 0.04 g SO_4^{2-}-S/g S^{2-} consumed. The phenol and sulfide oxidation was coupled to the denitrification process, obtaining a molecular nitrogen yield of 0.89 ± 0.06 g N_2/g NO_3^--N. NO_2^--N was detected in the effluent accounting for less than 0.8% of total NO_3^--N consumed. The mass balance for nitrogen, sulfur and carbon compounds was as follows:

168 mg $N_{Influent}$/l·d = [(150 mg N_2/l·d) + (1.2 mg NO_2^--N /l·d) + (9 mg suspended biomass-N/l·d)]$_{Effluent}$ = 160 mg N $_{Effluent}$ /l·d

168 mg $C_{Influent}$/l·d = [(133 mg HCO_3^- -C/l·d) + (3.6 mg $CO_{2\ (gas)}$ -C/l·d) + (23 mg suspended biomass-C/l·d)]$_{Effluent}$ = 160 mg C $_{Effluent}$ /l·d

37.8 mg $S^{2-}{}_{Influent}$/l·d = 35.42 mg SO_4^{2-}-$S_{Effluent}$ /l·d

The percentages of recuperation of nitrogen, carbon and sulfur products were 95, 95 and 94%, respectively from the substrates consumed.

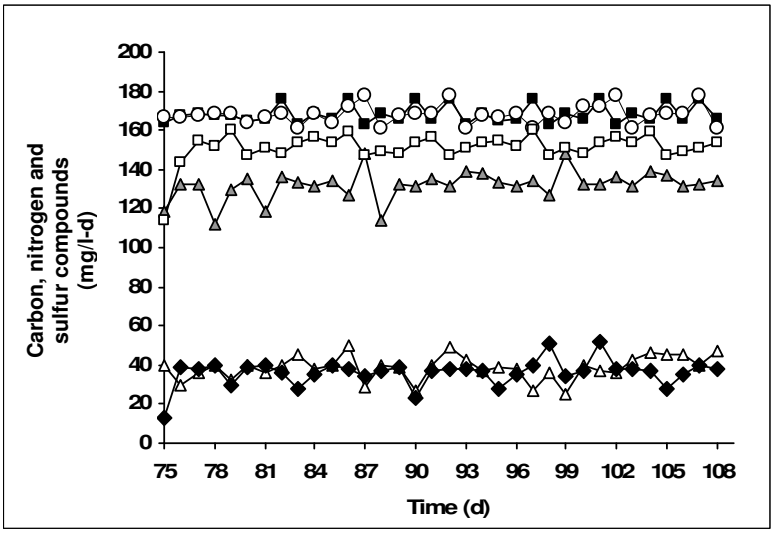

Figure 2 Profile of carbon, nitrogen and sulfur compounds under litho-organotrophic conditions operation in the inverse fluidized bed reactor. Influent NO_3^--N (■), phenol-C (○) and S^{2-} (Δ); effluent inorganic carbon (▲), SO_4^{2-}-S (♦) and N_2 gas (□).

The sulfide addition to the IFBR did not affect the respiratory process of denitrification. Thus, the microbial consortium used in this work was metabolically litho-organotrophic as was able to remove simultaneously both phenol and sulfide. Reyes *et al.* (2004) using an anaerobic stirrer tank reactor observed that sulfide and acetate oxidation was coupled to nitrate reduction to molecular nitrogen. However, in this case, the authors observed that sulfide was partially oxidized to elemental sulfur and the oxidation rate of sulfide was slower than acetate oxidation. In our work sulfide was completely oxidized to sulfate. Thomas et al. (2002) observed that the inhibition constant (*Ki*) for phenol oxidation was 113 mg/l under denitrifying conditions in a simple minimal mineral medium at an initial cell mass dry weight of 8 mg/l. In the present work the phenol concentration fed was above the inhibition constant. However, the phenol was totally consumed without intermediates accumulation. This high phenol consumption could be due to different factors such as phenol/biomass ratio, biomass attached onto a support plastic and a continuous reactor operation.

Microbial biofilm community analysis

Employing molecular biology techniques based on 16S rDNA were identified some chemolithotrophic microorganisms such as *Thiobacillus denitrificans*, *Thiobacillus sajanensis and Thiobacillus sp*., as is

shown in Table 1. Genera *Thiobacillus* are microorganisms well known to use reduced sulfur compounds (sulfide and thiosulfate) as energy and sulfur source. Sublette and Sylvester, (1987) showed that *Thiobacillus denitrificans* can grow in presence of heterotrophic microorganisms; such presence may have a positive effect diminishing organic waste products potentially inhibitory to *T. denitrificans*. Other studies showed that *T. denitrificans* was able to grow in presence of glucose or glutaraldehyde (Pan and Umbreit, 1972; Sublette and Woolsey, 1988). However, there are not evidences about the phenol consumption by *T. denitrificans*; thus indicating that heterotrophic microorganisms were involved in the phenol biodegradation. Several strains are known to be able of oxidizing phenol under denitrifying conditions, such as *Azoarcus sp*., *Thauera aromatica* K172 and strain S100 (Tschech and Fuchs, 1987; Anders 1995; Shinoda et al., 2000). Another strain is the mixotrophic bacterium *Thiosphaera panthotropha* which is able to denitrify using reduced sulfur compounds, hydrogen or a wide range of organic compounds as electron donors (Kuenen et al., 1992; Chazal and Lens, 2000). This strain has been found and isolated from a fluidized bed reactor fed with sulfide and acetate as electron donors under denitrifying conditions (Gommers et al., 1988). Thus, the simultaneous oxidation of phenol and sulfide coupled to the nitrate reduction could be carried out by the two different microbial genera.

Table 1 Some identified microorganisms on denitrifying biofilm

Strain no.	Closest phylogenetic relative	16S rDNA similitude	Phylogenetic group	*Accession no.*
ATCC 25259	*Thiobacillus denitrificans*	99%	*beta-Proteobacteria*	*CP000116*
1 HG	*Thiobacillus sajanensis*	98%	*beta-Proteobacteria*	*DQ390446*
44a-B2-21	*Thiobacillus sp.*	*98%*	*beta-Proteobacteria*	*AY082471*

CONCLUSION

The increase of phenol and sulfide loading rates to the IFBR did not affect the denitrification process. The mass balances indicated that phenol, sulfide and nitrate were completely removed, and the products were stoichiometrically recovered as bicarbonate, sulfate and N_2, respectively. Consequently, this study showed the potential of a denitrifying biofilm reactor for the treatment of complex industrial wastewater.

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CHAPTER 4

Sulfide oxidation under chemolithoautotrophic denitrifying conditions

ABSTRACT

Chemolithoautotrophic denitrifying microorganisms oxidize reduced inorganic sulfur compounds coupled to the reduction of nitrate as an electron acceptor. These denitrifiers can be applied to the removal of nitrogen and/or sulfur contamination from wastewater, groundwater and gaseous streams. This study investigated the physiology and kinetics of chemolithotrophic denitrification by an enrichment culture utilizing hydrogen sulfide, elemental sulfur or thiosulfate as electron donor. Complete oxidation of sulfide to sulfate was observed when nitrate was supplemented at concentrations equal or exceeding the stoichiometric requirement. In contrast, sulfide was only partially oxidized to elemental sulfur when nitrate concentrations were limiting. Sulfide was found to inhibit chemolithotrophic sulfoxidation, decreasing rates by approximately 21-fold when the sulfide concentration increased from 2.5 to 10.0 mM, respectively. Addition of low levels of acetate (0.5 mM) enhanced denitrification and sulfate formation, suggesting that acetate was utilized as a carbon source by chemolithotrophic denitrifiers. The results of this study indicate the potential of chemolithotrophic denitrification for the removal of hydrogen sulfide. The sulfide/nitrate ratio can be used to control the fate of sulfide oxidation to either elemental sulfur or sulfate.

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INTRODUCTION

Denitrification is an anaerobic microbial process in which nitrate (NO_3^-) is converted into the environmentally benign product, molecular nitrogen (N_2). Denitrification is often used as a means of eliminating nitrogen during wastewater treatment (Grady *et al.,* 1999). Most existing applications rely on heterotrophic microorganisms that utilize simple organic compounds such as methanol as an electron donating substrate to drive denitrification. Some denitrifying bacteria are chemolithoautotrophic and can oxidize inorganic sulfur compounds, such as sulfide (S^{2-}), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), or sulfite (SO_3^{2-}), anaerobically at the expense of the reduction of nitrate or other oxidized nitrogen acceptor compounds (Schedel and Truper, 1980; Timmer-ten Hoor, 1981; Kuenen *et al.,* 1992). Sulfur-utilizing chemolithoautotrophic denitrifiers are believed to play an important role in mineral cycling by linking sulfur and nitrogen cycles (Korom, 1992; Sørensen, 1987). Among these, two obligate autotrophic species are well known, *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, which grow at neutral pH (Schedel and Truper, 1980; Kuenen *et al.,* 1992; Timmer-Ten Hoor, 1975). Sulfoxidation by these microorganisms can proceed both under aerobic and under anoxic conditions. Oxidation of sulfide by chemolithoautotrophic denitrifying bacteria can lead to the formation of elemental sulfur or sulfate, depending on the physiological conditions. The stoichiometry of these reactions is shown below for sulfide in Eqs. (1) to (4). Comparison of Eqs. (1) and (2) shows that conversion to elemental sulfur coupled to complete denitrification consumes four times less nitrate as compared to complete oxidation to sulfate. Comparison of Eqs. (1) and (3) illustrates a 2.5-fold larger consumption of nitrate in reactions involving incomplete denitrification to nitrite.

$S^{2-} + 1.6\ NO_3^- + 1.6\ H^+ \rightarrow SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O$ $\Delta G°' = -743.9$ kJ/ reaction (1)

$S^{2-} + 0.4\ NO_3^- + 2.4\ H^+ \rightarrow S^0 + 0.2\ N_2 + 1.2\ H_2O$ $\Delta G°' = -191.0$ kJ/ reaction (2)

$S^{2-} + 4\ NO_3^- \rightarrow SO_4^{2-} + 4\ NO_2^-$ $\Delta G°' = -501.4$ kJ/ reaction (3)

$S^{2-} + NO_3^- + 2\ H^+ \rightarrow S^0 + NO_2^- + H_2O$ $\Delta G°' = -130.4$ kJ/ reaction (4)

The stoichiometry for the complete chemolithotrophic denitrification with elemental sulfur and thiosulfate is given below:

$S^0 + 1.2\ NO_3^- + 0.4\ H_2O \rightarrow SO_4^{2-} + 0.6\ N^2 + 0.8\ H^+$ $\Delta G°' = -547.6$ kJ/ reaction (5)

$S_2O_3^{2-} + 1.6\ NO_3^- + 0.2\ H_2O \rightarrow 2\ SO_4^{2-} + 0.8\ N_2 + 0.4\ H^+$ $\Delta G°' = -765.7$ kJ/ reaction (6)

Sulfide is corrosive, toxic, malodorous, and exerts an oxygen demand. Autotrophic denitrification is of interest to environmental technology because this biological process catalyzes the oxidation of sulfide and other reduced sulfur compounds (sulfoxidation) in the absence of elemental oxygen. The use of nitrate to control H_2S corrosion and odors in sewer systems has been known for many years and continues to be of commercial interest (Allen, 1949, Bentzen *et al.,* 1995, Carpenter, 1932). More recently, the addition of nitrate to sulfide-laden oil field brines was also shown to be an effective method to enhance the biological removal of sulfides and reduce problems associated with their toxicity, corrosivity and negative impact on reservoir permeability (Jenneman *et al.,* 1999, Reinsel *et al.,* 1996). Autotrophic denitrification has also been proposed for H_2S removal from biogas (Kleerebezem and Mendez, 2002). In wastewaters containing both N and S contaminants, nitrates generated after nitrification can be recirculated to anaerobic stages of the treatment process to promote the simultaneous oxidation of sulfides and denitrification of nitrates. The concept has been investigated for industrial wastewater treatment (Gommers *et al.,* 1988, Reyes-Avila *et al.,* 2004, Sierra-Alvarez *et al.,* 2005). Microbial transformation of hydrogen sulfide to elemental sulfur offers interesting opportunities for the removal of this contaminant. Elemental sulfur is insoluble and can be physically removed from effluents for reuse. Alternatively, hydrogen sulfide can be oxidized to sulfate for discharge where sulfate is environmentally benign (e.g., marine environment). Numerous processes based on the use of S^0 for the autotrophic denitrification of drinking water (Van der Hoek *et al.,* 1992, Darbi *et al.,* 2003) and wastewater (Gommers *et al.,* 1988, Nugroho *et al.,* 2002, Am *et al.,* 2005) are also reported in the literature. This study evaluates the kinetics of chemolithotrophic denitrification coupled to the oxidation of different reduced sulfur compounds including hydrogen sulfide, elemental sulfur and thiosulfate ($S_2O_3^{2-}$). The main focus of the study was to investigate the role of sulfide and nitrate levels on the kinetics and stoichiometry of chemolithoautotrophic denitrification. The research utilized an enrichment culture obtained from a continuous denitrifying bioreactor fed with thiosulfate as electron donor. These results provide clues on how sulfide oxidation can be steered to either sulfate (for direct discharge) or elemental sulfur (for recovery) as end products.

MATERIALS AND METHODS

Enrichment Culture

The chemolithotrophic denitrifying enrichment culture was cultivated in a 2 L upward flow anaerobic sludge bed (UASB) reactor operated over a period of 2 months at a temperature of 30 ± 2˚C with an empty bed hydraulic retention time averaging 24 h. The reactor was fed an influent (pH 7.0) containing near stoichiometric concentrations of thiosulfate (18.3 to 41.3 mM) and nitrate (29.2 to 76.0 mM) in a basal mineral medium composed of (g/L): K_2HPO_4 (0.80), KH_2PO_4 (0.30), NH_4Cl (0.40), $MgCl_2$ (0.01), $NaHCO_3$ (2.00), and trace elements solution supplied at 2 mL/L. The trace element solution contained (g/L): EDTA (0.50); $ZnSO_4{\cdot}7H_2O$ (0.04); $CaCl_2{\cdot}2H_2O$ (0.07); $MnCl_2$ (0.03); $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.01); $CuSO_4{\cdot}H_2O$ (0.02); $CoCl_2{\cdot}6H_2O$ (0.02). The bioreactor was inoculated with 10 g volatile suspended solids (VSS) per liter of methanogenic granular sludge. The inoculum was obtained from a full scale UASB reactor treating recycle paper effluent (Industriewater, Eerbeek, The Netherlands). The VSS content of the chemolithotrophic cultivated in the bioreactor and the methanogenic sludge used as bioreactor inoculum was 7.5% and 12.9%, respectively. Sludge samples were washed and sieved to remove fine particles before use in the tests. The microbial cultures were stored under nitrogen gas at 4°C.

Batch Bioassays

Batch experiments were conducted in glass serum flasks (165 ml) supplemented with 100 mL of medium. The medium (pH 7.0) contained basal mineral nutrients, as described above for the enrichment culture, and variable concentrations of nitrate (supplemented as KNO_3) and sulfide (supplemented as $Na_2S\ 7H_2O$). Experiments conducted to assay chemolithotrophic denitrification with thiosulfate (as $Na_2S_2O_3$) and powdered elemental sulfur were supplied with 2.50 and 3.33 mM of these electron donors, respectively, and stoichiometric concentrations of nitrate (4 mM). The experiments were inoculated with 0.5 g VSS/L of the chemolithotrophic culture, unless otherwise indicated. The headspace was flushed with helium: CO_2 mixture (80:20, v/v) to exclude oxygen from the assays. All flasks were sealed with butyl rubber stoppers and aluminum crimp seals. Separate bottles were set up for gas phase measurements of N_2. Controls lacking inoculum were run in parallel to monitor the possible abiotic degradation of the electron donor and electron acceptor. Controls lacking NO_3^- or S^{2-} were also included to correct for compound losses not associated with

chemolithotrophic denitrification. All bioassays were carried out in triplicate and were incubated in a climate-controlled chamber at 30 ± 2˚C in an orbital shaker at 150 r.p.m. The results reported are average values of three replicates. Standard deviation values were under 5%.

Liquid samples were withdrawn periodically to monitor the conversion of substrates and the formation of degradation products. Sulfide was analyzed immediately thereafter to prevent compound losses by volatilization and/or abiotic oxidation. Samples for nitrate determination were membrane filtered (0.45 µm). The initial pH value in all assays was 7.0 and final pH values ranged from 7.1 to 7.3. The specific sulfide oxidation and denitrifying activities were calculated from the slope of the sulfide and nitrate concentration; respectively, versus time (d), and are reported in mmol NO_3^- /g VSS d, and mmol S^{2-}/g VSS d, respectively. Headspace samples in the bioassays were analyzed for molecular nitrogen (N_2) and nitrous (N_2O) gas content at regular intervals throughout the experiment.

Analytical Methods

Nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) were determined by ion chromatography (IC) with suppressed conductivity detection using a Dionex DX-500 system equipped with an AS11-HC Dionex column (Dionex, Sunnydale, CA). The mobile phase was KOH at a flow rate of 1.2 mL/min. The eluent gradient of KOH utilized was: 1 mM at 0 min; 2 mM at 5.5 min and 8.5 mM at 12 min. The injection volume was 25 µL. Sulfide was analyzed colorimetrically by the methylene blue method (Trüper and Schlegel, 1964). Molecular nitrogen (N_2) and nitrous (N_2O) gas content in headspace samples was analyzed by gas chromatography using an HP5290 Series II system (Agilent Technologies, Palo Alto, CA) equipped with a thermal conductivity detection. The gas chromatograph was fitted with a CarboxenTM 1010 Plot capillary column (30 m x 0.32 mm, Supelco, St. Louis, MO). The temperature of the column, the injector port and the detector was 100, 220 and 230°C, respectively. The carrier gas was helium at a flow rate of 45 mL/min and a split flow of 15 mL/min. Headspace samples (100 µL) were collected using a pressure-lock gas syringe. The pH was determined immediately after sampling with an Orion model 310 PerpHecT pHmeter with a PerpHecT ROSS glass combination electrode. VSS were determined according to standard methods (APHA, 1999).

RESULTS

Chemolithotrophic denitrification coupled to oxidation of different reduced sulfur Compounds

The rates of chemolithotrophic denitrification in assays utilizing different reduced sulfur compounds as electron donors were compared. The average oxidation state of the sulfur atoms in the three compounds tested, *i.e.,* sulfide, elemental sulfur and thiosulfate, is –2, 0 and +2, respectively. Figure 1 illustrates the conversion of nitrate and the formation of nitrogen containing products as a function of time for the various assays. Also plotted in this figure is the time course of conversion for the various reduced sulfur compounds to sulfate.

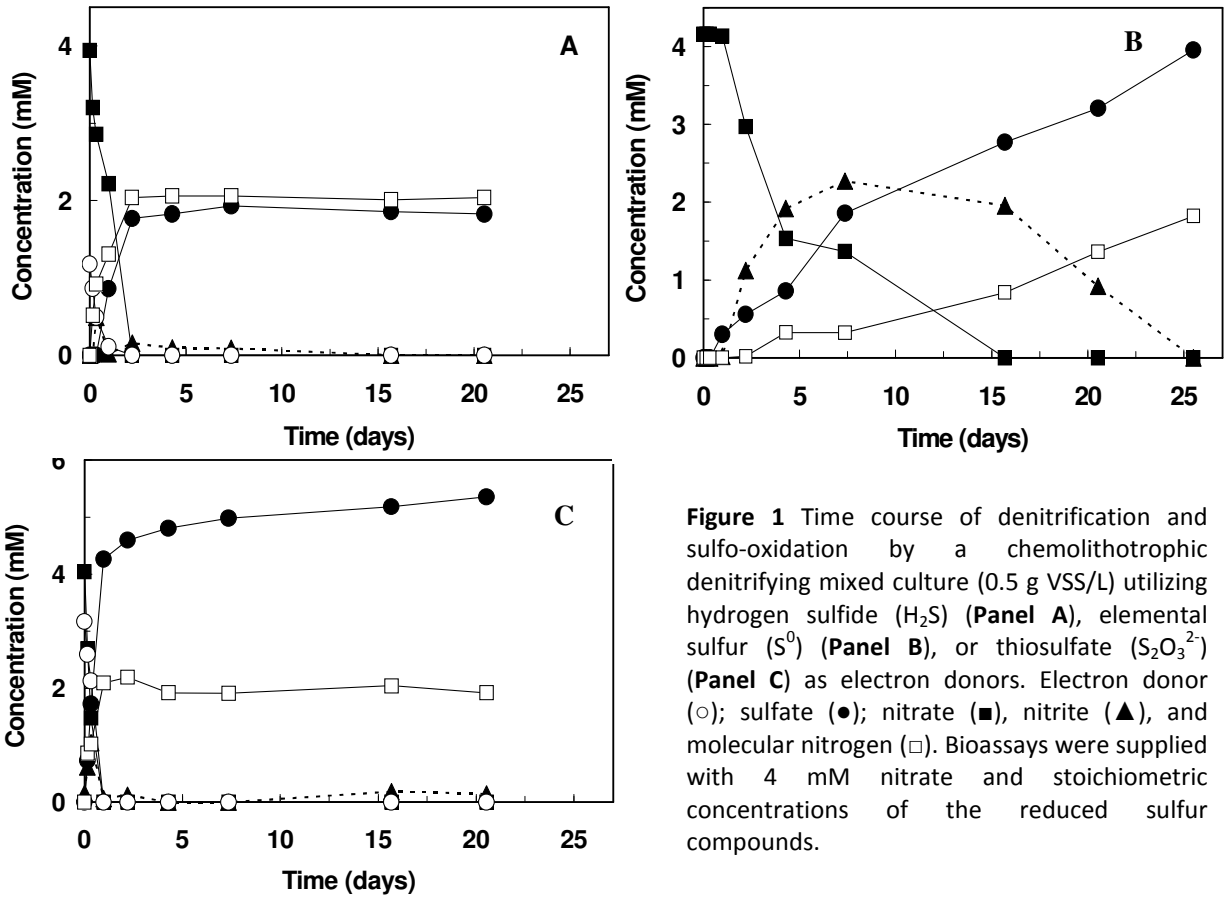

Figure 1 Time course of denitrification and sulfo-oxidation by a chemolithotrophic denitrifying mixed culture (0.5 g VSS/L) utilizing hydrogen sulfide (H_2S) (**Panel A**), elemental sulfur (S^0) (**Panel B**), or thiosulfate ($S_2O_3^{2-}$) (**Panel C**) as electron donors. Electron donor (○); sulfate (●); nitrate (■), nitrite (▲), and molecular nitrogen (□). Bioassays were supplied with 4 mM nitrate and stoichiometric concentrations of the reduced sulfur compounds.

Nitrogenous gas intermediates were not detected in this experiment. Table 1 list the maximum rates of nitrate degradation and sulfate generation determined in the experiments with the different electron donors. Thiosulfate was the most readily utilized electron donor, followed by hydrogen

sulfide and elemental sulfur. The rates of nitrate degradation in assays with thiosulfate were 4.6 and 9.5 fold higher compared to sulfide and elemental sulfur, respectively. Similarly, the rates of sulfate generation in assays with thiosulfate were 4.8 and 25.3-fold higher compared to sulfide and elemental sulfur, respectively.

Table 1 Rates of nitrate conversion and sulfate generation by a chemolithotrophic denitrifying enrichment culture utilizing different reduced sulfur compounds as electron donating substrate.

	Nitrate degradation rate (mM NO_3^-/d)	**Sulfate generation rate (mM SO_4^{2-}/d)**	**Final stoichiometry (mol N_2/mol SO_4^{2-})**	***Theoretical stoichiometry (mol N_2/mol SO_4^{2-})***
Thiosulfate	*7.56 ± 0.04*	*4.33 ± 0.08*	*0.39*	*0.40*
Sulfide	*1.65 ± 0.03*	*0.91 ± 0.04*	*0.86*	*0.80*
Elemental Sulfur	*0.80 ± 0.03*	*0.17 ± 0.04*	*0.45*	*0.60*

Nitrite was either not detected or only at very low concentrations in the cultures supplied with thiosulfate and hydrogen sulfide. In contrast, accumulation of nitrite at relatively high concentrations (up to 2.3 mM) was observed in the experiments with elemental sulfur. However, nitrate was recovered as N_2 gas in (near) stoichiometric proportions by the end of the experiments. Similarly, stoichiometric conversion of elemental sulfur to sulfate was observed in the different assays (Table 1), as demonstrated by the final N_2/ SO_4^{2-} ratios being close to the expected values.

Effect of nitrate concentrations on chemolithotrophic denitrification

Batch bioassays were conducted to evaluate the impact of increasing nitrate levels (0, 1, 4 and 16 mM) on the rates of anoxic sulfide oxidation (2.5 mM S^{2-}). Based on the stoichiometry presented in Eq. (1), 1 mM NO_3^- is insufficient for total oxidation of sulfide to sulfate; whereas 4 mM NO_3^- is the stoichiometric requirement, and 16 mM NO_3^- provides a large excess of electron accepting capacity. Figure 2 and 3 shows the time course of nitrogen and sulfur conversion, respectively, in the bioassays. In cultures supplied with 1 mM NO_3^-, accumulation of NO_2^- was observed during the initial phase of the experiment. Nitrite concentration decreased steadily after day 2, coinciding with an increase of the volume of N_2 recorded (Figure 2). The yield of nitrogen as N_2 (0.40 mmol per liter of culture medium) and the residual level of nitrite (0.14 mM) account for a recovery of 0.94 mM N, which is in

good agreement with the amount of nitrate consumed (1.0 mM). Nitrite accumulation followed by complete denitrification to N_2 gas was also observed in the assays with 4 and 16 mM NO_3^- (Figure 2). The final recovery of nitrogen as N_2, 1.95 and 2.18 mmol N_2/L, respectively, was in agreement with the observed sulfoxidation.

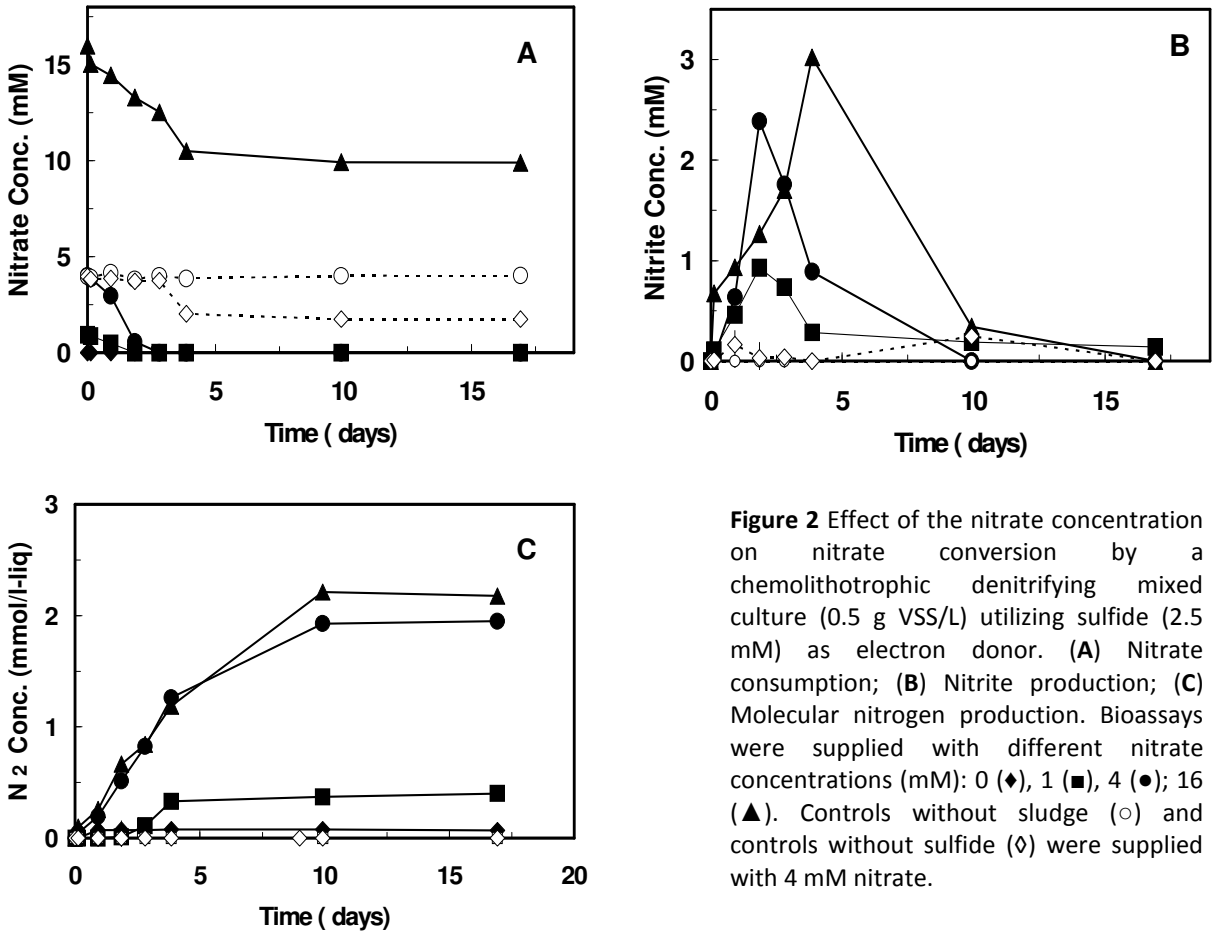

Figure 2 Effect of the nitrate concentration on nitrate conversion by a chemolithotrophic denitrifying mixed culture (0.5 g VSS/L) utilizing sulfide (2.5 mM) as electron donor. (**A**) Nitrate consumption; (**B**) Nitrite production; (**C**) Molecular nitrogen production. Bioassays were supplied with different nitrate concentrations (mM): 0 (♦), 1 (■), 4 (●); 16 (▲). Controls without sludge (○) and controls without sulfide (◊) were supplied with 4 mM nitrate.

The initial S^{2-}/NO_3^- ratio impacted the end products from sulfide oxidation. Sulfide was quantitatively recovered as sulfate in the presence of stoichiometric or excess nitrate concentrations (Figure 3). In contrast, when nitrate was limiting (1 mM), sulfide oxidation led to the formation of an unidentified intermediate, and sulfate was not detected. The medium acquired a white turbid color; suggesting partially oxidation of sulfide to elemental sulfur (colloidal form). This result is in agreement with Eq. (2), since 1 mM NO_3^- is theoretically required to oxidize 2.5 mM S^{2-} to elemental sulfur. A small

decrease in nitrate in controls lacking sulfide was not due to denitrification since no N_2 or nitrite was formed.

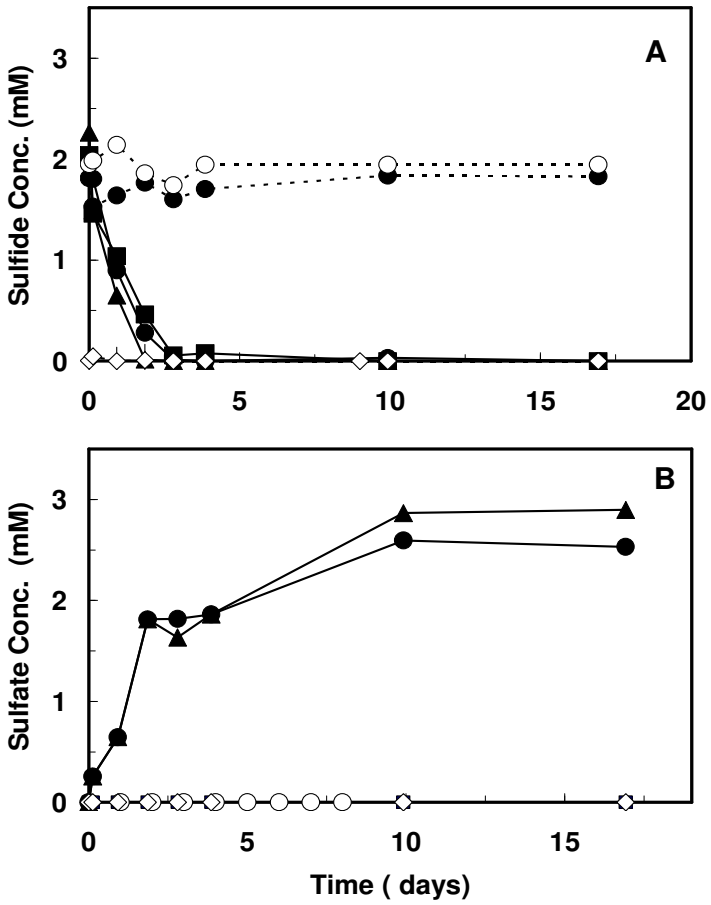

Figure 3 Effect of the nitrate concentration on sulfide conversion by a chemolithotrophic denitrifying mixed culture (0.5 g VSS/L) utilizing sulfide (2.5 mM) as electron donor. (**A**) Sulfide consumption; (**B**) Sulfate production. Bioassays were supplied with different nitrate concentrations (mM): 0 (♦), 1 (■), 4 (●); and 16 (▲). Controls without sludge (○) and controls without sulfide (◊) were supplied with 4 mM nitrate.

Figure 4 shows that the rate of sulfoxidation increased asymptotically with increasing nitrate concentrations and it approached a maximum (1.75 mmol /g VSS d) at nitrate levels of 4 mM and higher. The rate of nitrate consumption also increased linearly with nitrate concentration up to 4 mM, and thereafter partially decreased.

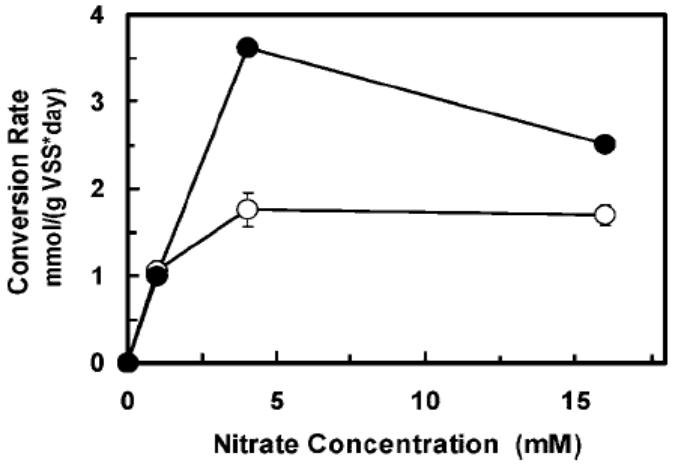

Figure 4 Rate of nitrate (●) and sulfide (○) removal by a chemolithotrophic denitrifying mixed culture (0.5 g VSS/L) as a function of the initial nitrate concentration during batch bioassays supplied with 2.5 mM sulfide.

Effect of sulfide concentrations on chemolithotrophic denitrification

The impact of increasing sulfide levels on the rates of anoxic denitrification by a chemolithotrophic consortium was evaluated in bioassays supplied with 8 mM nitrate. Based on the stoichiometry presented in Eq. (1), 5 mM sulfide are required for complete reduction of nitrate to N_2.

Figure 5 illustrates the conversion of nitrate and sulfide as a function of time. The maximum specific denitrification rate decreased by approximately 21-fold from 1.61 to 0.07 mmol NO_3^-/g VSS d when the sulfide concentration changed from 2.5 to 10 mM, respectively (Figure 6). The sharp decrease in the rate of denitrification and sulfate generation suggests that sulfide was toxic to the chemolithotrophic denitrifying microorganisms. The highest denitrification rate was determined in the assays with 2.5 mM sulfide, in spite of the fact that sulfide was limiting. Nitrate was fully depleted in the assay with 5 mM, albeit at lower rates compared to the treatment containing only 2.5 mM of this compound. Low denitrification rates were observed during the initial phase of the assay with 7.5 mM sulfide, but the treatment eventually recovered after sulfide was depleted on day 7, which was also reflected in an increase of the rates of denitrification and sulfate production. Very low denitrification rates were observed at 10 mM sulfide. In this treatment, denitrification was not paralleled by sulfate production, except for a low rate of sulfate production after 11 days.

Nitrite was detected as a temporal biotransformation intermediate in the assays supplied with 2.5 to 7.5 mM sulfide at concentrations up to 3.8 mM. No nitrite was detected in the assay with 10 mM sulfide. The delay for nitrite appearance increased with increasing initial H_2S concentration.

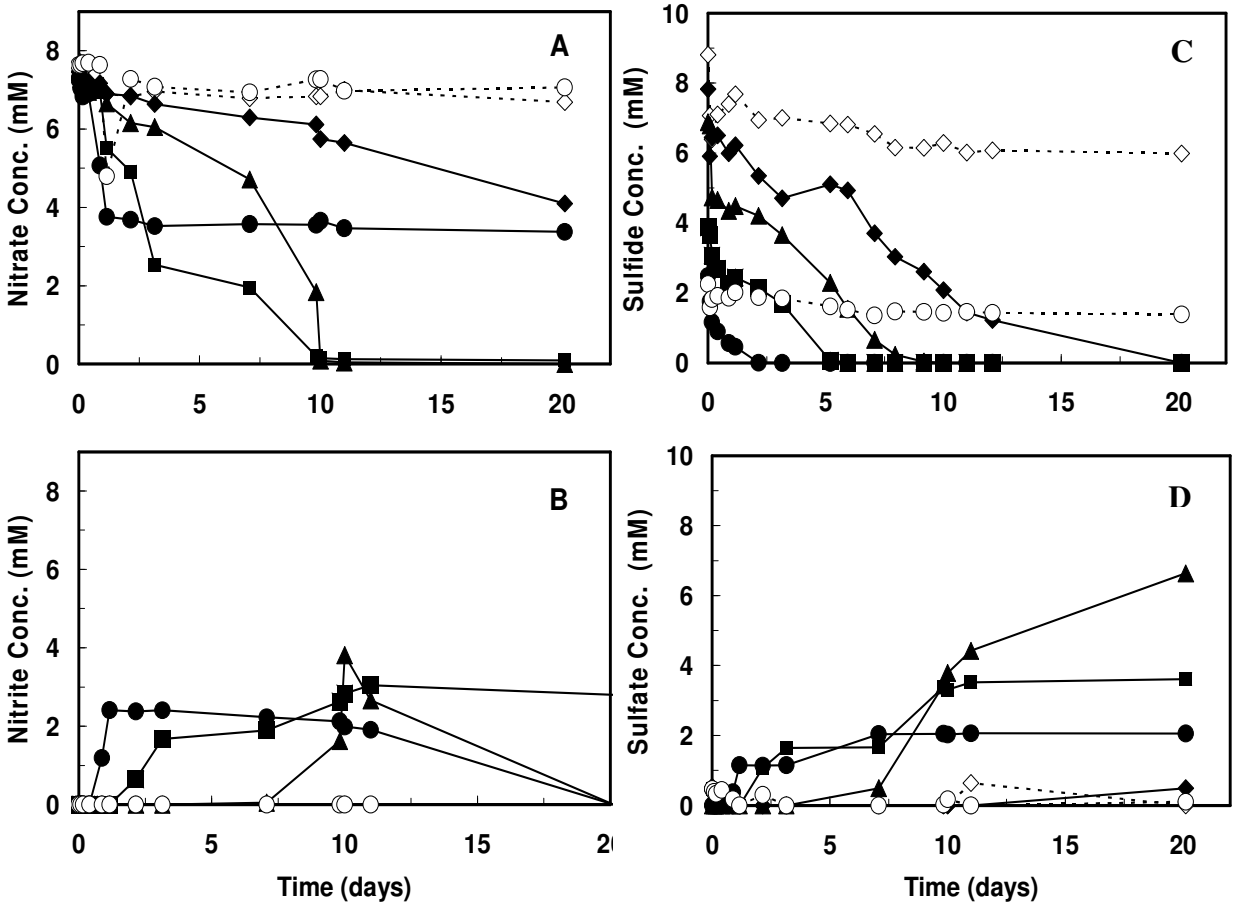

Figure 5 Effect of the sulfide concentration on nitrate and sulfide conversion by a chemolithotrophic denitrifying mixed culture (2 g VSS/L) supplied with 8.0 mM nitrate. **(A)** Nitrate consumption; **(B)** Nitrite production; **(C)** Sulfide consumption; and **(D)** Sulfate production. Bioassays were supplemented with different sulfide concentrations (mM): 2.5 (●); 5.0 (■); 7.5 (▲); and 10.0 (♦). Two uninoculated controls were also included for comparison, one with 2.5 mM sulfide (◊) and other with 10 mM sulfide (○).

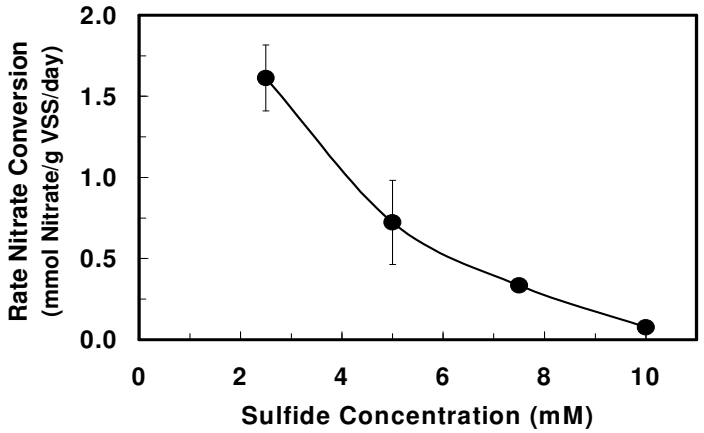

Figure 6 Rate of specific denitrification by a chemolithotrophic mixed culture as a function of the initial sulfide concentration during batch bioassays supplied with 8 mM nitrate.

Effect of acetate on chemolithotrophic denitrification

The effect of adding a small amount of acetate (0.5 mM) on denitrification and sulfoxidation by the chemolithotrophic sludge is shown in Figure 7. The initial concentration of nitrate and sulfide in the assays was 10 and 2.5 mM, respectively. The acetate addition significantly decreased the lag phase of denitrification as evidenced by nitrate consumption and sulfate formation data. The rates of denitrification and sulfate formation were also enhanced by 23.3 and 25.3%, respectively, in the presence of acetate. Acetate alone (without sulfide) only supported marginal denitrification in agreement with the limited electron donor supply.

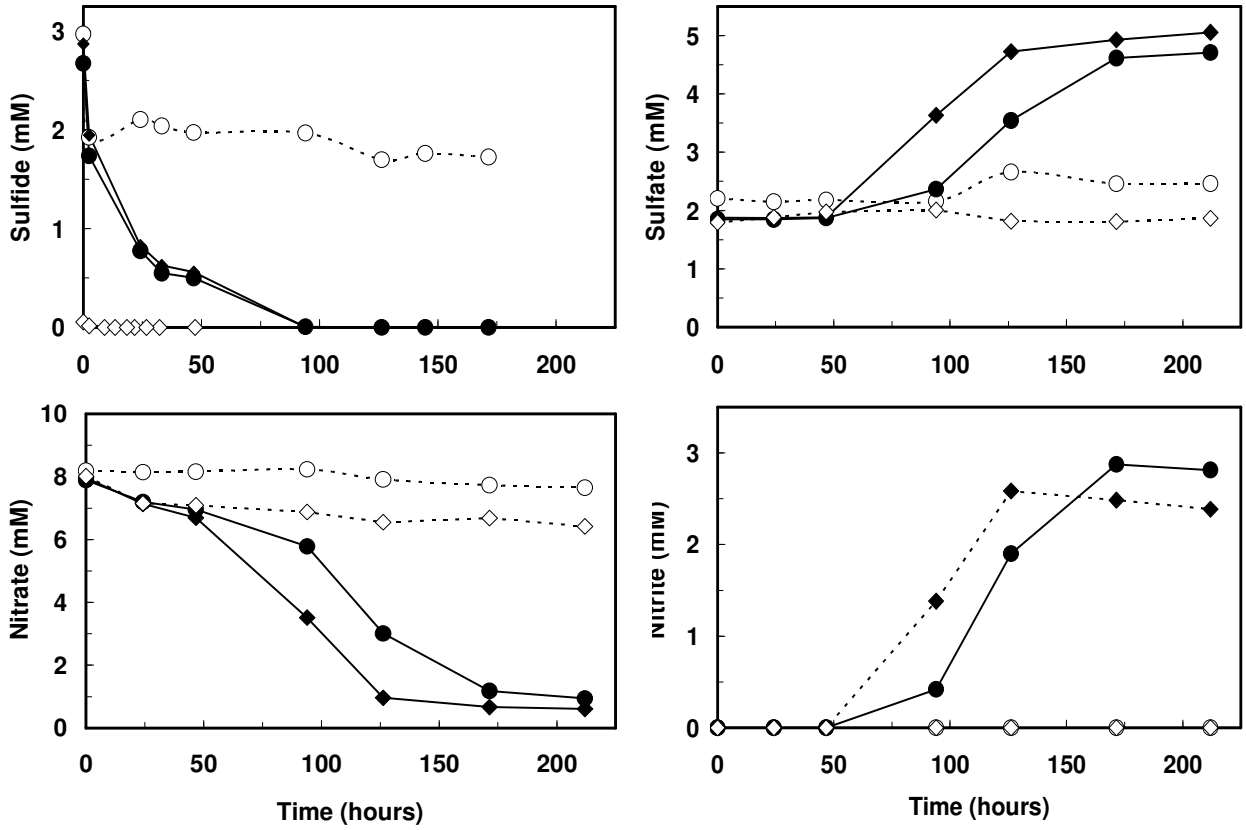

Figure 7 Effect of acetate (0.5 mM) on the conversion of nitrate (10 mM) and sulfide (2.5 mM) by a chemolithotrophic denitrifying mixed culture. Elimination of sulfide (**Panel A**); formation of sulfate (**Panel B**); elimination of nitrate (**Panel C**); formation of nitrite (**Panel D**). Treatments: No acetate added (●); 0.5 mM acetate (♦); uninoculated control, no acetate addition (○); uninoculated control with 2.5 mM acetate and 0 mM sulfide (◊).

DISCUSSION

Chemolithotrophic denitrification coupled to oxidation of different reduced sulfur compounds

The metabolic activity of the chemolithotrophic denitrifying enrichment culture was considerably higher with thiosulfate compared to sulfide or elemental sulfur (Table 1). Thiosulfate is readily bioavailable and non-toxic, which could partly explain the high sulfoxidation and denitrification rates detected with this compound. While, hydrogen sulfide is also bioavailable, it is a well-known inhibitor of a wide variety of microorganisms, including denitrifying bacteria (Sorensen *et al.,* 1980; Brunet and Garcia-Gil, 1996), and its inhibitory impact may account for lower metabolic rates compared to thiosulfate.

The lowest rates were observed for chemolithotrophic denitrification of elemental sulfur and this is most likely due to the limited mass transfer of substrate from solid phase S^0 (Beristain-Cardoso *et al.,* 2005). Elemental sulfur is an apolar mineral, thus mass transfer is expected to be an important rate-limiting factor in the overall process. The specific surface area of elemental sulfur is a principal factor governing the kinetics of its biological oxidation (Konishi *et al.,* 1995, Tichy *et al.,* 1994), including oxidation linked to denitrification (Koenig and Liu, 2001).

Effect of nitrate concentrations on chemolithotrophic denitrification

The dependence of the rate of H_2S oxidation on the concentration of nitrate (Figure 4) appears to follow Michaelis–Menten enzyme kinetics, with an estimated maximum velocity of 1.75 mmol/d and a half velocity constant of 0.66 mM. Nitrate consumption rate, on the other hand, decreased somewhat at the highest nitrate concentrations tested (Figure 4) and this may be due to inhibition by nitrate or accumulated nitrite. Nitrate was reported to inhibit chemolithotrophic denitrification by denitrifying sulfur bacteria when present at high concentrations of 47 mM (Oh *et al.,* 2000).

The NO_3^-/S^{2-} ratio had a strong impact on the formation of intermediate and final degradation products by the chemolithotrophic culture. Sulfide was quantitatively recovered as sulfate in assays supplied with nitrate levels equal or exceeding stoichiometric requirements. The ratios of $N_{2\ formed}/SO_4^{2-}{}_{formed}$ obtained at the end of the batch experiments ranged from 0.75 to 0.86, which are consistent with the theoretical value of 0.80 for the complete oxidation of sulfide linked to denitrification (Eq. 1). Likewise the ratios of $NO_3^-{}_{consumed}/SO_4^{2-}{}_{formed}$ were for the most part in the range of 1.45 to 1.58, which are also consistent with the theoretical value of 1.60 (Eq. 1). In contrast, when nitrate was limiting, sulfate was either not detected at all or only partially formed. The most limited supply of electron acceptor was tested in an experiment in which the initial concentrations of H_2S and NO_3^- were 2.5 and 1 mM, respectively. The electron equivalents of NO_3^- only provided enough oxidant for 25% of the H_2S. Under these extreme conditions no sulfate was formed, instead the formation of S^0 was assumed to be the final product. There are three lines of evidence pointing to the occurrence of elemental sulfur and/or polysulfides. Firstly, the formation of a white/yellowish colloidal precipitate was observed which is in keeping with the insoluble nature of S^0. Secondly, there was a complete loss of the soluble sulfur species monitored, H_2S, $S_2O_3^{2-}$ and SO_4^{2-}. Thirdly, the measured stoichiometric ratios of $NO_3^-{}_{consumed}/H_2S_{\ consumed}$ (0.46) and of $N_{2\ formed}/H_2S_{\ consumed}$ (0.19) are consistent

with denitrification linked to the partial oxidation of H_2S to S^0 (Eq. 2). Krishnakumar and Manilal (1999) observed the same behavior under nitrate-limited conditions in batch experiments where the solution became white probably due to accumulation of S^0 as intermediate of sulfide oxidation. Based on literature reports, elemental sulfur is an expected product of the partial oxidation of sulfide (Kelly *et al.,* 1997). Elemental sulfur has also been reported to accumulate in chemolithotrophic denitrifying bioreactors treating sulfide during periods of sulfide overloading (Gommers *et al.,* 1988; Reyes-Avila *et al.,* 2004; Krishnakumar and Manilal, 1999). Similarly, elemental sulfur is the major product of the aerobic oxidation of sulfide when oxygen is limited (< 0.1 mg/L) (Buisman *et al.,* 1990), while sulfate is formed under sulfide limitation (Buisman *et al.,* 1993). These results suggest that partial oxidation from sulfide to S^0 is driven by a limitation of electron acceptor.

Even in cases where there was a better supply of electron acceptor, the occurrence of elemental sulfur and/or polysulfides was frequently observed as transient products in the pathway to sulfate. This can be best witnessed by observing a temporal hole in the sulfur balance during the duration of the assay as shown in Figure 8 for two typical experiments.

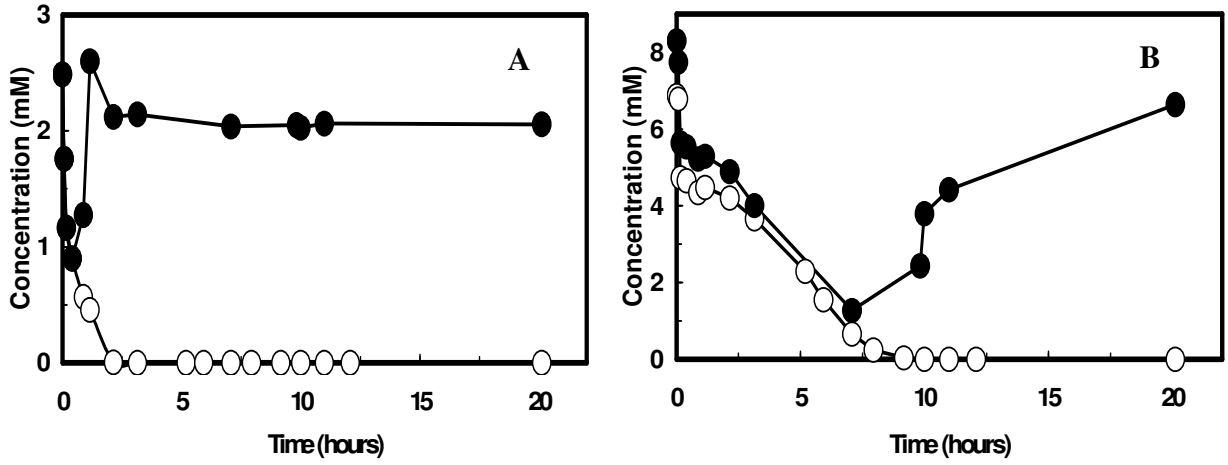

Figure 8 Concentration of sulfide (○) and total concentration of sulfur species (sulfide + sulfate + thiosulfate) (●) detected in chemolithotrophic denitrifying experiments supplied with 8.0 mM nitrate and either 2.5 mM H_2S (Panel A) or 7.5 mM H_2S (Panel B), as a function of time.

When the gap in the sulfur balance was at its peak, the $NO_3^-{}_{consumed}/H_2S_{consumed}$ ratios of 0.33 to 0.46 are observed which are in agreement with a theoretical value of 0.40 for the partial oxidation of H_2S to S^0 linked to denitrification (Eq. 2). The gap in the sulfur balance also coincided with the occurrence of turbidity. Ratios between 0.30 and 0.40 would be expected if some elemental sulfur reacted with

H_2S to form polysulfides (Kleinjan *et al.,* 2003). Figure 7 shows that eventually the S-balance is restored; coinciding with the formation of sulfate and measured stoichiometric ratios of $NO_3^-{}_{consumed}/H_2S_{consumed}$ that approach the value of 1.60, expected for the complete oxidation of sulfide (Eq. 1).

Effect of sulfide concentrations on chemolithotrophic denitrification

Sulfide at concentrations ranging from 2.5 to 10 mM was found to cause increasing inhibition of nitrate conversion and sulfate generation (Figure 6). Sulfide has been shown earlier to exert inhibitory effects towards denitrifying heterotrophic (Knowles, 1982; Schonharting *et al.,* 1998) and chemolithotrophic microorganisms (Gommers *et al.,* 1988; Sublette *et al.,* 1998). Several studies considering the impact of sulfide on heterotrophic denitrification have reported strong inhibition of nitrous oxide reduction (Sorensen *et al.,* 1980, Schonharting *et al.,* 1998). It is unclear whether nitrous oxide reductase was inhibited since nitrogenous gas intermediates were not quantified in this experiment. Nonetheless, our results clearly indicate that sulfide inhibited the conversion of nitrate to nitrite. The fact that nitrite accumulation was delayed or did not occur at all at 7.5 and 10 mM S^{2-}, respectively, is due most likely to a delay in the degradation of nitrate. Nitrite conversion did not appear to be severely affected by sulfide as evidenced by the relatively similar values determined for the maximum concentration of nitrite in the various treatments (Figure 4). The practical implication of these findings is that treatment of sulfide-laden wastewaters by chemolithoautotrophic denitrification should be operated under conditions ensuring that the effective steady-state sulfide concentration in the reactor is below strongly inhibitory levels.

It is noteworthy that the rate of sulfide elimination was not greatly affected by sulfide even at the highest concentration tested (Figure 5). The rapid conversion of sulfide might be explained by the low electron equivalent requirement for the oxidation of sulfide to elemental sulfur coupled to the reduction of nitrate to N_2, 0.40 mol sulfide/mol nitrate (Eq. 2). Abiotic mechanisms such as the formation of polysulfides (S_x^{2-}) by reaction of biologically produced S^0 particles with dissolved H_2S will also contribute to additional removal of sulfide. Eq. (7) illustrates the stoichiometry for the reaction of S_8 elemental sulfur, a typical configuration of sulfur generated by sulfur oxidizing bacteria such as *T. denitrificans* (Kleinjan *et al.,* 2003), with hydrogen sulfide.

$$HS^- + ((x-1)/8)\ S_8 \rightarrow S_x^{2-} + H^+ \qquad (7)$$

At neutral to intermediate alkalinities, S_6^{2-} , S_5^{2-} and S_4^{2-} ions dominate in solution.

Effect of acetate on chemolithotrophic denitrification

The stimulatory effect of acetate on shortening the lag phase of autotrophic denitrification (Figure 8) and enhancing denitrification rates may be due to the use of acetate as a carbon source by chemolithotrophic denitrifiers in the mixed culture. Acetate is expected to be assimilated more readily than inorganic carbon, and thus support higher cell yields. Sulfoxidizing denitrifiers commonly detected in natural environments and engineering systems, such as *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, are strict chemolithoautotrophs (Kuenen, 1979).

However, numerous facultative chemolithoautotrophs capable of fixing CO_2 and assimilating available acetate when sulfur or sulfide is present as an energy source have been described, such as *Thiobacillus* (now *Paracoccus*) v*ersutus*, *Thiosphaera* (now *Paracoccus*) *pantotropha, Paracoccus denitrificans*, *Thioploca* and *Beggiatoa* spp. (Kuenen *et al.,* 1992; Hagen and Nelson, 1997; Otte *et al.,* 1999). These bacteria are very versatile and can adapt to autotrophic, heterotrophic, or mixotrophic conditions (Matin, 1978).

These results indicate that addition of small amounts of organic carbon to the feed of chemolithotrophic denitrification bioreactors could be advantageous to enhance treatment and maintain a more robust bioreactor system compared to strict autotrophic denitrification. Biological treatment systems promoting simultaneous autotrophic and heterotrophic denitrification have interesting applications in the combined removal of nitrogen, sulfur and organic contamination from wastewaters (Sierra-Alvarez *et al.,* 2005; Oh *et al.,* 2003).

CONCLUSIONS

The rates of denitrification and sulfoxidation determined for a chemolithotrophic enrichment culture depended strongly on the inorganic sulfur compound utilized as electron donor (thiosulfate > sulfide >> elemental sulfur). Furthermore, the results point to the possibility of controlling the fate of sulfide oxidation to either elemental sulfur or sulfate by manipulating the nitrate/sulfide ratios in the medium. A sub-stoichiometric dose of nitrate could be used to promote partial oxidation to elemental sulfur as opposed to complete oxidation to sulfate. The study confirms that high sulfide concentrations are inhibitory to chemolithotrophic denitrification, particularly affecting the

conversion of nitrate to nitrite. Finally, the organic carbon source, acetate, was shown to stimulate chemolithotrophic denitrification by the sulfoxidizing bacteria enrichment culture.

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CHAPTER 5

Chemolithotrophic denitrification with elemental sulfur for groundwater treatment

ABSTRACT

Nitrate is an important groundwater pollutant. This study examined the use of a biological treatment process to remove nitrates in groundwater that relies on the activity of autotrophic denitrifying microorganisms that utilize elemental sulfur (S^0) as electron donor and limestone as inorganic carbon source. A packed-bed reactor supplied with sulfur:limestone granules (1:1, v/v) was rapidly started up utilizing a chemolithotrophic denitrifying enrichment culture as inoculum. After 30 days, 100% NO_3^- removal efficiency was achieved at a high volumetric loading rate of 161 mg NO_3^--N/L d. The recovery of nitrogen as benign N_2 gas was nearly stoichiometric. The results of this study indicate the potential of autotrophic denitrification with elemental sulfur for the treatment of nitrate in groundwater.

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INTRODUCTION

Nitrate in groundwater originates primarily from agricultural fertilizers, septic systems, landfills, mining and wastewater treatment plants. Nitrate is not significantly attenuated by the soil and it is transported to the groundwater largely unchanged. Nitrate concentrations exceeding the maximum contaminant level allowed (10 mg/L of NO_3^--N) can cause methemoglobinemia or "blue-baby disease" (Gangolli *et al.,* 1994). In adults, high nitrate levels have been associated with cancer (NAS, 1977). With the rapid population increase in arid regions, and the concomitant increase in water demands, feasible technologies for treating nitrate-contaminated groundwater are becoming increasingly important. Physical-chemical methods such as reverse osmosis and ion exchange are costly, require chemical additives and generate waste brines. As an alternative, it is proposed the use of a biological treatment process to convert nitrates to benign N_2 gas with elemental sulfur as the reducing agent. The proposed process does not require dosing of expensive electron donors and does not generate waste brines.

Denitrification is an anaerobic microbial process in which nitrate (NO_3^-) is converted into molecular nitrogen (N_2) in four enzymatic steps via the intermediates nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (Zumft, 1997). The ability to respire nitrate under anaerobic conditions is widespread among several genera of heterotrophic bacteria. Heterotrophic denitrifiers utilize simple organic substances such as methanol, ethanol and glucose, as electron donating substrates. Some denitrifying bacteria are chemolithoautotrophic and use reduced sulfur compounds such as elemental sulfur (S^0), sulfide (S^{2-}), thiosulfate ($S_2O_3^{2-}$), or sulfite (SO_3^{2-}) as electron donors (Schedel and Trüper, 1980; Timmer-ten-Hoor, 1981). Under chemolithoautotrophic conditions, carbon dioxide or bicarbonate are used as a carbon source for microbial cell synthesis.

A technology under consideration that utilizes S^0 for the biological conversion of nitrates to harmless molecular nitrogen is the “Sulfur − Limestone Autotrophic Denitrification” (SLAD) process. The elemental sulfur serves as the electron-donor to support chemolithoautotrophic denitrification. The process generates acidity as indicated by the following stoichiometric reaction:

$$NO_3^- + \frac{5}{6}S^0 + \frac{1}{3}H_2O \rightarrow \frac{1}{2}N_2 + \frac{5}{6}SO_4^{2-} + \frac{2}{3}H^+ \quad (1)$$

Limestone serves to buffer the generated acidity as well as to supply inorganic carbon for cell synthesis by the denitrifying bacteria. Dutch scientists first proposed the SLAD technology in the year 1987 (Schippers *et al.,* 1987). Since then, several studies have reported on its applicability for the removal of nitrate in drinking water (Flere and Zhang, 1999; Koenig and Liu, 2002; Darbi *et al.,* 2003). The objective of this study was to evaluate the role of elemental sulfur and nitrate concentrations on the kinetics and stoichiometry of autotrophic denitrification by a granular sludge enrichment culture cultivated with nitrate and thiosulfate. An additional objective was to demonstrate a rapid start-up of the SLAD process in a bioreactor utilizing a granular sludge enrichment culture as inoculum.

MATERIALS AND METHODS

Enrichment Culture

An autotrophic denitrifying granular sludge enrichment culture was cultivated in a 2 L upward flow anaerobic sludge bed (UASB) reactor over a period of 2 months utilizing a feed containing 42.2 mM thiosulfate, 80.4 mM nitrate and mineral nutrients. The reactor was initially inoculated with 10 g volatile suspended solids (VSS)/L anaerobic granular sludge obtained from a full-scale UASB reactor treating recycle paper effluent in Eerbeek, The Netherlands.

Batch Experiments

Batch experiments were conducted in 160 mL serum flask containing 25 mL of medium. The medium (pH 7) contained variable concentrations of powdered S^0, 4.3 mM of NO_3^-. The basal mineral medium utilized was composed of (g/L): K_2HPO_4 (0.80); KH_2PO_4 (0.30); NH_4CI (0.40); $MgCl_2$ (0.01); $NaHCO_3$ (2.00); and trace elements solution supplied at 2 mL/L. The trace element solution contained (g/L): EDTA (0.50); $ZnSO_4 \cdot 7H_2O$ (0.04); $CaCl_2 \cdot 2H_2O$ (0.07); $MnCl_2$ (0.03); $(NH_4)_6Mo7O_{24} \cdot 4H_2O$ (0.01); $CuSO_4 \cdot H_2O$ (0.02); $CoCl_2 \cdot 6H_2O$ (0.02). The experiments were inoculated with 0.5 g VSS/L of the enrichment culture. The headspace was flushed with He:CO_2 mixture (80/20, v/v). Duplicate bottles for each treatment were incubated at 30°C on an orbital shaker (150 rpm). Separate bottles were set up for gas phase measurements of N_2. Controls lacking NO_3^- or sludge were also tested.

Continuous Experiments

A packed bed reactor (0.4 L) was filled with a mixture of S^0 granules (120.8 ml), limestone grit (128.4 ml) between 5 and 16 mesh, and then was inoculated with 1.2 g VSS/L of enrichment culture granular

sludge (Figure 1). A thin layer of of small glass spheres (diameter 3 mm) was supplied in the lower part of the reactor to facilitate retention of the reactor packing and preven clogging of influent lines. The S^0 granule particle size was approximately 3.5 mm wide $\times$ 1 mm thick. The total mass of S^0 added to the reactor was 141.3 g. The reactor was fed with an influent containing 7.1 mM NO_3^-, and basal mineral medium containing (g/L): KH_2PO_4, 1; $MgSO_4 \cdot 6H_2O$, 0.2; NH_4Cl, 0.4; Na HCO_3, 2; trace element solution (described above), 2 ml/L. Two HRT were assayed 1.24 and 0.65 days. A peristaltic pump (Gilson Minipuls III, Middleton, WI) was use to feed the mineral medium in to the reactor. The production of molecular nitrogen and N_2O was measured for liquid displacement using an inverted 1 L glass serum flask filled with a 3% (w/v) NaOH solution to scrub out the carbon dioxide from the biogas. The conversion of substrates was monitored periodically by measuring the influent concentration of nitrate; the concentration of nitrate, nitrite, sulfate, thiosulfate and sulfide in the efluent. Fresh efluent samples were collecte from the efluent line. Gas samples obtained from the headspace of the scrubber were analyzed weekly for N_2 an N_2O content.

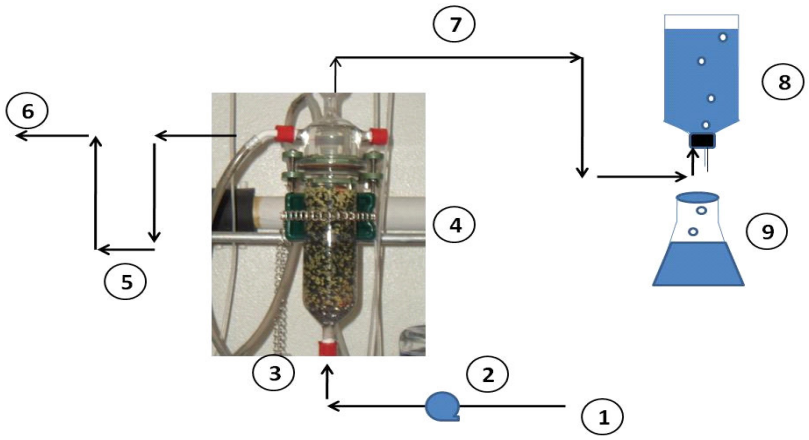

Figure 1 Schematic representation of the continuous flow bioreactor utilized in this study. (1) Reactor feed; (2) peristaltic pump; (3) glass beads; (4) sulfur–limestone-granular sludge bed; (5) water lock; (6) treated effluent; (7) gas; (8) inverted flask filled with 3% NaOH (w/v) for scrubbing carbon dioxide and/or sulfide from the gas, and for monitoring gas production; (9) Erlenmeyer to collect the liquid displaced from the scrubber.

Analytical Methods

Nitrate, nitrite, sulfate and thiosulfate were determined by ion chromatography with suppressed conductivity detection using a Dionex DX-500 system equipped with Dionex CD conductivity detector,

an AS11-HC Dionex column (4mm x 250mm) and an AG16 guard column (4mm x 40mm) (Dionex, Sunnydale, CA). The eluent gradient of KOH utilized was: 1mM at 0min; 2mM at 5.5min and 8.5mM at 12min, and the flow rate 1.2mL/min. The injection volume was 25 mL. Liquid samples were membrane filtered (0.20 µm) prior to chromatographic analysis. Sulfide in liquid samples was analyzed immediately after sampling to prevent compound osses by volatilization and/or abiotic oxidation. Sulfide was quantified colorimetrically by the methylene blue method (Truper and Schlegel, 1964). N_2 and N_2O in gaseous samples were analyzed by gas chromatography using an HP5290 Series II system (Agilent Technologies, Palo Alto, CA) fitted with a TM Carboxen 1010 Plot capillary column (30m x 0.32mm, Supelco, St. Louis, MO) and with a thermal conductivity detector. The temperature of the column, the injector port and the detector was 100, 220 and 230 °C, respectively. The carrier gas was helium at a flow rate of 45mL/min and a split flow of 15mL/min. Headspace samples (100 mL) were collected using a pressure-lock gas syringe. The pH was determined mmediately after sampling with an Orion model 310 PerpHecT pH-meter with a PerpHecT ROSS glass combination electrode. VSS were determined according to Standard Methods for the Examination of Water and Wastewater (APHA 1998).

RESULTS AND DISCUSSION

Impact of elemental sulfur concentration on denitrification activity

Elemental sulfur is an insoluble, apolar mineral, thus mass transfer is expected to be an important rate-limiting factor in the overall process. Metabolic activity of the granular biofilm enrichment culture used for the inoculation of the sulfur-based denitrification bioreactor at variable concentrations of powdered elemental sulfur (0, 1.9, 3.8, 7.6 and 15.2 mM) was tested in batch assays. Based on the stoichiometry presented in Eq. (1), a sulfur concentration of 3.8 mM would meet the theoretical requirement for complete reduction of the nitrate (4.3 mM) supplemented in the various assays to N_2. Detailed results are shown for the treatments that received 1.9 and 15.2 mM S^0 in Figure 2. With 12.9 mM S^0, nitrate was slowly converted to both nitrite and N_2 but the reaction ceased when only about half of the nitrate was consumed in accordance with the less than stoichiometric supply of electron donor. With 15.2 mM S^0, nitrate was rapidly and completely converted to N_2. Nitrite was detected as an intermediate degradation product during the initial phase of the assay, but was later depleted. The recovery of nitrogen as N_2 and/or nitrite and the formation of sulfate at the end of the experiments were in good agreement with the theoretical yields

determined based on the consumption of nitrate. In all treatments, thiosulfate and N_2O were periodically monitored but were never detected as intermediates. The rate of denitrification increased linearly with S^0 concentration up to 7.6 mM, which was a large excess of S^0 with respect to nitrate supplied (Figure 3). Beyond 7.6 mM, the rate continued to increase but no longer in a linear fashion. In the absence of elemental sulfur, an endogenous rate of 0.47 mmol NO_3^-/g VSS d was measured due to electron donors in the sludge.

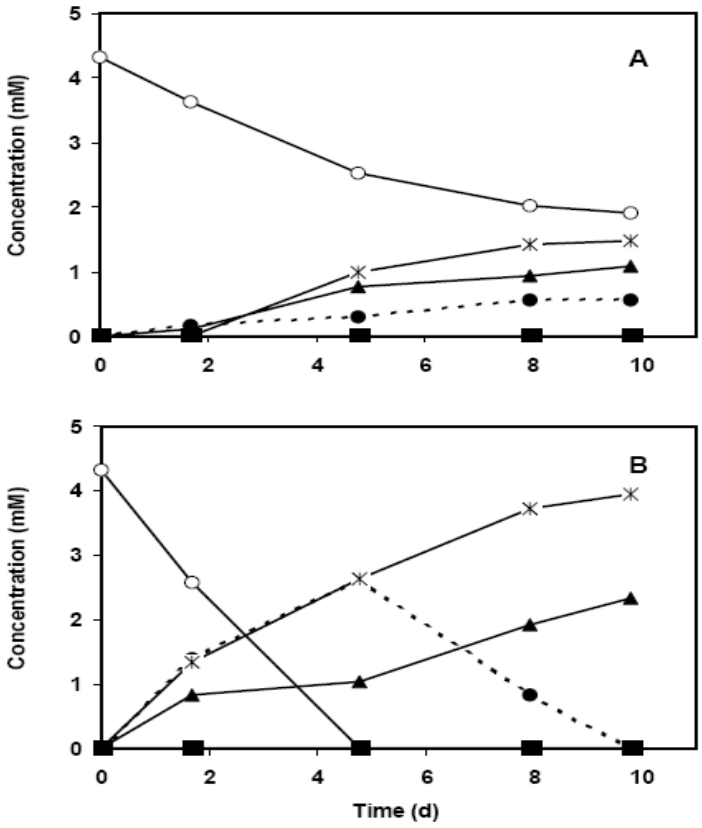

Figure 2 Chemolithotrophic denitrification with elemental sulfur. The effect of elemental sulfur concentration on the rate of denitrification and products formed by chemolithotrophic denitrifying sludge (0.5 g VSS/L). (Panel A). 1.9 mM elemental sulfur. (Panel B). 15.2 mM elemental sulfur. Concentrations (mmol/L of liquid) of: (○) Nitrate; (●) Nitrite; (▲) N_2; (X) Sulfate.

The endogenous rate, however, was only sustained for 5 d, at which time the nitrate level remained constant at 3.2 mM. The presence of S^0 substantially increased the rate beyond the endogenous rate and, in all cases; the S^0 enhanced the extent of denitrification. A continued increase in denitrification rates at concentrations far exceeding the stoichiometric requirement was probably due to limited

mass transfer from solid-phase S^0. Elemental sulfur is an apolar mineral, which is relatively insoluble in water. The maximum aqueous solubility of elemental sulfur is 0.16 mM (Steudel and Holdt, 1998). Thus, mass transfer should be expected to be a factor limiting autotrophic denitrification. The increased surface area provided at higher S^0 concentrations most likely resulted in better mass transfer, as will be discussed below.

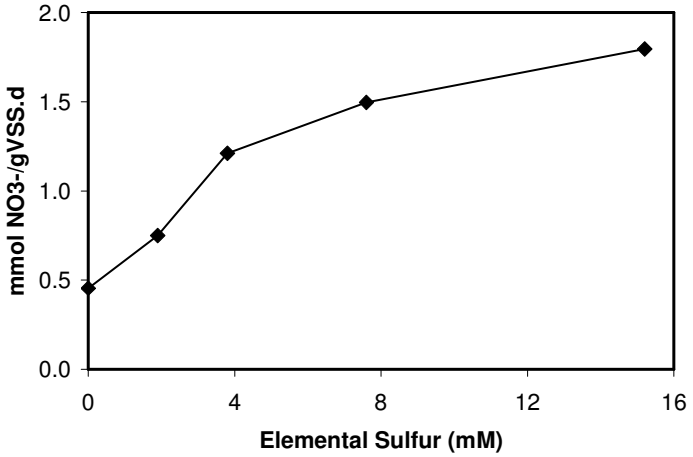

Figure 3 Relationship between the specific rate of denitrification and the concentration of elemental sulfur in batch bioassays inoculated with chemolithotrophic denitrifying sludge (0.5 g VSS/L) and supplied with 4.3 mM nitrate.

Continuous bioreactor experiment

The results from the continuous experiment are shown in Figure 4. For the first 30 days, nitrate was loaded at approximately 5.8 mmol /L d (81 mg NO_3^--N/L d). Afterwards the volumetric loading rate was increased to 11.5 mmol/L d (161 mg NO_3^--N/L d). At both loadings, complete removal of nitrate was observed to non-detect levels in the effluent. The recovery of N as N_2 gas was nearly stoichiometric and no NO_2^- was detected providing evidence for complete denitrification. Based on the N_2 evolved, the theoretical discharge of SO_4^{2-} was 4.9 and 10.6 mmol/L d in the first and the second period, respectively. These values were in close agreement with the measured values in the first period and slightly lower than the measured values in the second period (12.2 mM/L d). The small 8.6% discrepancy in the second period is attributed to experimental errors.

The results suggest that inoculation of the bioreactor with the enrichment culture sludge permitted the rapid start up of the SLAD process. The loading rate of 161 mg NO_3^--N/L d obtained with 100%

NO_3^- removal efficiency is comparable with the highest loading rates obtained with the SLAD process in the literature. Darbi *et al*. (2003) provided a load of 111 mg NO_3^--N/L d and obtained 92% or higher efficiency of NO_3^- removal. Flere and Zhang (1999) applied loading rates between 175 to 225 mg NO_3^--N/L d and attained a NO_3^- removal efficiency of 95%.

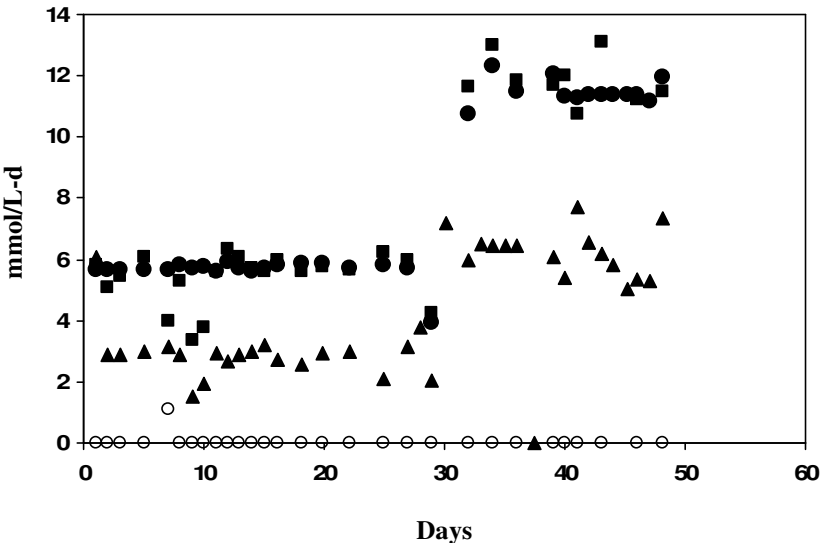

Figure 4 Nitrate removal in the laboratory-scale sulfur limestone autotrophic denitrification (SLAD) bioreactor inoculated with the enrichment culture. Nitrate in the influent (●); Molecular nitrogen (▲); nitrate in the effluent (○); Sulfate in the effluent (■).

Sulfide has been detected in effluents from sulfur-based denitrification reactors at concentrations of up to 0.31 mM (Van der Hoek *et al.,* 1992). In our experiments, sulfide was monitored periodically and the measured concentrations were generally under the detection limit (1.5 mM). Sulfide was only detected in a few samples, albeit at very low concentrations of 6.6 mM or lower. Formation of sulfide in autotrophic denitrification reactors is likely due to microbial disproportionation of sulfur (Thamdrup *et al.,* 1993). Production of sulfide is undesirable due to the toxic, corrosive, malodorous and O_2-consuming properties of this contaminant. In addition to sulfide, traces of assimilable organic carbon (1.7-12.5 mM) (Hijnen *et al.,* 1988; Kimura *et al.,* 2002) and increased microbial counts (103-104 colony forming units/L) (Flere and Zhang, 1998) have been reported in the treated water from sulfur-based denitrification processes. Therefore, a post-treatment step such as cascade aeration followed by sand filtration will possibly be required to polish the treated effluent.

CONCLUSIONS

Elemental sulfur can readily be utilized under anoxic conditions as an electron donor to support denitrification. The rates of denitrification were shown to increase with sulfur concentration, even when supplied in great stoichiometric excess. This observation can be explained by the increase in surface area at greater sulfur concentrations, and the ensuing improvement in mass transfer from insoluble elemental sulfur. Due to the poorly soluble nature of elemental sulfur, denitrification could become kinetically limited at high nitrogen volumetric loadings. A packed bed reactor with an approximate ratio 1:1 of sulfur:limestone granules was rapidly started up utilizing a chemolithotrophic denitrifying enrichment culture as inoculum. After 30 days, 100% NO_3^- removal efficiency was achieved at a loading of 161 mg NO_3^--N/L d, comparable to the fastest rates achieved in the literature with S^0 as electron donor. Taken together, the results of this study indicate the potential of autotrophic denitrification with elemental sulfur for the remediation of nitrate at the concentrations typically found in contaminated groundwater and surface water.

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GENERAL CONCLUSIONS

The main conclusions with regard to the present work are:

A complete oxidation of sulfide and phenol was coupled to the nitrate reduction when nitrate was supplemented at stoichiometric concentrations in batch cultures. The sulfide and phenol oxidation was sequential, first sulfide was oxidized to sulfate, and phenol was successively consumed. It was found that the sulfur compounds were faster oxidized than phenol. When initial sulfide concentration was increased up to 32 mg/L no changes in the specific rates of sulfide oxidation were observed, however, denitrifying metabolism on phenol oxidation was affected as N_2O was accumulated and a decreasing of the specific phenol and nitrate consumption rate was seen. It was observed that the stoichiometric definition of nitrate concentration was important for oxidizing completely phenol and sulfide, avoiding inhibition problems. These results showed that is possibly to remove simultaneously two compounds: phenol and sulfide, which can be found in petrochemical wastewaters.

The increase of phenol and sulfide loading rates to the IFBR did not affect the denitrification process. The mass balances indicated that phenol, sulfide and nitrate were completely removed, and the products were stoichiometrically recovered as inorganic carbon, sulfate and N_2. By mean of the analysis of 16S rRNA of the denitrifying consortium it could be identified the genera *Thiobacillus denitrificans*, *Thiobacillus sp*. and *T. sajanensis*. This denitrifying sludge showed a litho- and organotrophic metabolism, as it was oxidized either organic or inorganic compound. Consequently, this study showed the potential of a denitrifying biofilm reactor for the treatment of complex industrial wastewater.

In batch cultures under lithotrophic conditions was seen that the rates of denitrification and sulfoxidation depended strongly on the inorganic sulfur compound utilized as electron donor (thiosulfate, sulfide and elemental sulfur). This study confirmed that high sulfide concentrations are inhibitory to lithotrophic denitrification, as nitrite was accumulated. The results provided evidence that sulfide oxidation can be oriented either sulfate (for direct discharge) or elemental sulfur (for recovery) as end products.

The elemental sulfur could readily be utilized under denitrifying conditions as an electron donor. It was observed that the rates of denitrification increased with sulfur concentration, even when it was

supplied in excess. The use of elemental sulfur instead of organic matter as electron donor is a feasible alternative to remove nitrate from groundwater. The packed bed reactor with an approximate ratio 1:1 of sulfur:limestone granules removed NO_3^- as the removal efficiency was of 100% using a HRT of 15 h. The results of this study indicate that the lithotrophic denitrification can be used for the remediation of ground and surface water.

From the results of this work it is possible to say that the denitrification can be applied in two ways:

1) organo-lithotrophic process, which can be used for removing compounds from complex wastewaters such as the ones of chemical and petrochemical industry, and

2) lithotrophic process, where the contaminated water does not have organic matter such as groundwater and surface water.

RECOMMENDATION

In order to apply the denitrification to the wastewater from petrochemical industry it is necessary make more basic studies. Regarding to this, it is required to evaluate the effect of a mixture of phenolic compounds on denitrification process as well as to evaluate different loading rates. This work must be associated to more basic physiological studies and possibly to microbial analysis. Nevertheless, if we want to treat the petrochemical wastewater, it is indispensable to design a sequential treatment, as the residual water of the petrochemical industry does not contain nitrate. The effluent of petrochemical industry contains phenolics compounds, sulfide and ammonia. Thus, it is necessary a nitrifying reactor in order to oxidize the ammonia to nitrate, which could be recycled to a denitrifying reactor for oxidizing sulfide and phenolic compounds. A denitrification/nitrification configuration for the treatment of this complex wastewater is presented in Figure A. There are experimental evidences suggesting that the nitrification process is inhibited by sulfide and phenol, it is then first necessary to remove sulfide and phenol during the denitrification adding external nitrate. Thus, in the denitrifying reactor sulfide and phenol will be oxidized up to sulfate and bicarbonate, respectively, coupled to the nitrate reduction to molecular nitrogen. Afterward, the residual ammonia and the product of denitrification such as sulfate and bicarbonate will be fed to the nitrifying reactor where the ammonia will be oxidized. The nitrate, sulfate and bicarbonate will be recycled to the denitrifying IFBR to carry out the denitrification process.

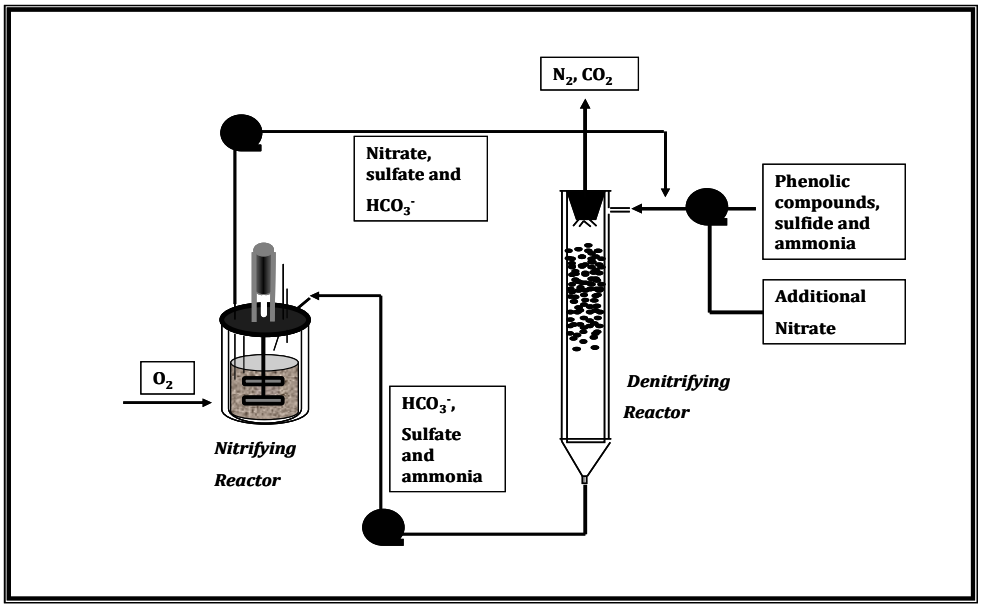

Figure A Proposed configuration for removing compounds from petrochemical industry.