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Fluidized-bed bioreactor applications for the treatment of metal-, sulfate- and nitrate-contaminated mine waters

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ABSTRACT

Acid mine drainage (AMD) is an important environmental problem related to the release of acidic, sulfate and metal-containing wastewater into the environment. It contaminates groundwaters and thousands of kilometers of streams in many countries all over the world. Sulfate and metal contamination in AMD is often associated with nitrogen pollution, especially due to the use of N-based explosives in mining and extractive industry. In scientific literature, several bioreactor configurations have been aimed at metal, sulfate and nitrate removal from wastewaters. Among those, the fluidized-bed reactor (FBR) is very efficient for AMD remediation due to the high biomass retention, the possibility to use high loading rates at low hydraulic retention times (HRT), the great resistance to inhibitors and the potential of recycling the produced pH buffered water to maintain neutral conditions in the reactor.

In this work, FBRs and batch assays were used to study metal depletion kinetics, sulfate reduction and nitrate removal. Metal sulfide recovery from bioreactors is as important as metal depletion. The influence of sulfide concentration and nutrients commonly present in mineral media and wastewaters on Zn, Cu, Pb and Cd precipitation kinetics and characteristics was evaluated in batch experiments. When sulfide was fed stoichiometrically or in excess, metals precipitated within 9 hours. On the contrary, when sulfide was below the stoichiometric metal to sulfide ratio, the metals with slower depletion rates (Zn and Cd) were susceptible to other removal mechanisms such as biosorption onto the sulfate reducing biofilm.

A sulfidogenic process was developed for treating acidic sulfate-containing wastewater in two inverse fluidized-bed reactors (IFBR). The process was based on sulfate reduction by sulfate-reducing bacteria (SRB) and neutralization of the water with biologically produced bicarbonate alkalinity. Low-density

polypropylene pellets were used as biomass carrier and lactate was chosen as electron donor for sulfate reduction. Two different COD/sulfate ratios were used for the operation of the reactors.

During the 242 days of operation, the robustness of the system was studied by suddenly decreasing the feed pH. A 10% fluidization degree was used since the carrier material adopted showed not to be adequate to attain a satisfactory immobilization of the biomass with higher fluidization degrees. This resulted in a failure of the process when the feed pH was intentionally decreased to 3. On the contrary, when a slightly acidic feed solution was fed, a 97% sulfate reduction efficiency was obtained with a COD/sulfate ratio of 4. With a stoichiometric COD/sulfate ratio, COD removal and sulfate reduction efficiencies reached the highest values of 75% and 35%, respectively. Higher efficiencies were not achieved due to the accumulation of acetate and the presence of different microbial species competing for lactate.

Denitrification of acidic water was investigated in two up-flow FBRs and using batch assays as well. Bacterial communities were enriched on ethanol plus nitrate in the FBRs. The effects of temperature, pH and ethanol/nitrate ratio on denitrification were revealed. Denitrification in FBR was maintained at 7-8°C and feed pH of 2.5. Batch assays revealed that a feed pH of 3 was inhibitory to denitrification. In FBRs, nitrate and ethanol were removed and the feed pH was neutralized, provided that ethanol was supplied in excess to nitrate. The use of stoichiometric nitrate to ethanol ratio resulted in complete ethanol oxidation and 66% and 76% nitrate removal at 7-8°C and 22°C, respectively. Polymerase chain reaction - denaturant gradient gel electrophoresis demonstrated the co-existence of different denitrifying microbial consortia. *Dechloromonas denitrificans* and *Hydrogenophaga caeni* were present in both FBRs and mainly responsible for nitrate reduction.

SOMMARIO

L’acid mine drainage (AMD) costituisce un importante problema di natura ambientale associato allo scarico nell’ambiente di acque reflue generalmente caratterizzate da forte acidità ed elevate concentrazioni di solfati e metalli pesanti. I suoi effetti colpiscono sia le acque di falda che le acque superficiali in numerosi Paesi del mondo. Tale contaminazione da solfati e metalli pesanti spesso si accompagna ad un inquinamento da eccessivo scarico di composti dell’azoto, soprattutto a causa del largo utilizzo di esplosivi a base di azoto nell’industria mineraria.

Metalli, solfati e nitrati possono essere rimossi dalle acque reflue utilizzando processi biologici. In letteratura, numerose configurazioni impiantistiche di bioreattori sono state utilizzate ai fini della rimozione di queste sostanze. Fra queste, i reattori a letto fluido (FBR) costituiscono una tecnologia molto promettente per il trattamento delle acque reflue da attività minerarie in quanto: i) trattengono la biomassa in maniera molto efficiente, ii) permettono di utilizzare alti fattori di carico e bassi tempi di detenzione idraulica (HRT), iii) presentano una grande resistenza ad agenti inibenti e iv) favoriscono condizioni di neutralità di pH ai microrganismi grazie al ricircolo della portata già trattata e alla diluizione della portata in ingresso.

Nel presente lavoro di tesi, sono stati condotti studi sulle cinetiche di rimozione dei metalli, sulla solfato riduzione e sulla rimozione dei nitrati all’interno di reattori del tipo FBR ed esperimenti “batch”. Il recupero di metalli, sotto forma di solfuri, dai bioreattori è importante tanto quanto la loro precipitazione e rimozione. L’influenza della concentrazione di solfuro e di nutrienti, solitamente presenti in acque minerali e reflue, sulle cinetiche di precipitazione di Zn, Cu, Pb and Cd e sulla natura dei cristalli formatisi è stata valutata nell’ambito di prove batch. È stato osservato che, quando i solfuri erano presenti in quantità

uguale o superiore a quella stechiometrica, Zn, Cu, Pb and Cd precipitavano entro 9 ore dall'inizio dell'esperimento. Al contrario, con concentrazioni di solfuro inferiori a quella stechiometrica, i metalli caratterizzati da una cinetica più lenta come Zn e Cd venivano rimossi secondo differenti meccanismi quale l'adsorbimento sul biofilm.

Un processo biologico di solfato riduzione è stato sviluppato in due reattori inversi a letto fluido (IFBR) per il trattamento di acque fortemente acide e con elevate concentrazioni di solfati. Attraverso l'utilizzo di batteri solfato-riduttori (SRB) è possibile, oltre a ridurre i solfati a solfuri, anche neutralizzare il pH della portata in ingresso ai reattori grazie all'alcalinità prodotta per via biologica. Piccole sfere di polipropilene sono state utilizzate come supporto per la crescita e l'attecchimento della biomassa mentre l'acido lattico è stato scelto come donatore di elettroni. I due reattori hanno funzionato con due differenti rapporti COD/solfati.

Durante i 242 giorni di esercizio dei reattori, la robustezza del processo di solfato riduzione è stata studiata diminuendo bruscamente il pH della portata influente. Il grado di fluidizzazione utilizzato è stato pari al 10% dato che, dopo pochi giorni dalla messa in esercizio dei reattori, il materiale di supporto utilizzato si è dimostrato non adatto per l'attecchimento della biomassa a gradi di fluidizzazione più elevati. Questo ha portato ad una totale inibizione del processo quando il pH in ingresso era pari a 3. Al contrario, quando il pH della portata in ingresso era pari a 5, un'efficienza di solfato riduzione del 97% è stata ottenuta alimentando uno dei reattori con un rapporto COD/solfati pari a 4. Con un rapporto stechiometrico tra COD e solfati pari a 0.67, invece, le efficienze di rimozione del COD e dei solfati si sono attestate al 75% e al 35% rispettivamente. Efficienze più alte non sono state ottenute per via della formazione di acetato, come intermedio di reazione della solfato riduzione, e la

presenza di altre specie microbiche in competizione con i solfato-riduttori per l'acido lattico.

Il processo di denitrificazione di acque acide è stato anch'esso studiato in due reattori del tipo "upflow-FBR" ed in esperimenti batch. L'accrescimento batterico è avvenuto utilizzando etanolo come fonte di carbonio e i nitrati come accettori di elettroni. Gli effetti del pH in ingresso, della temperatura e del rapporto etanolo/nitrati sul processo di denitrificazione sono stati valutati. Gli esperimenti batch hanno mostrato che un valore di pH pari a 3 è inibente per l'attività dei microrganismi denitrificanti. Nei reattori, invece, il processo è avvenuto con successo anche a valori di pH in ingresso pari a 2.5 e ad una temperatura di 7-8°C. Con etanolo presente in soluzione in eccesso rispetto ai nitrati, etanolo e nitrati sono stati consumati e il pH influente è stato riportato su valori neutri. Con etanolo aggiunto in rapporto stechiometrico rispetto ai nitrati, l'efficienza di rimozione dei nitrati è stata pari al 66% e al 76%, rispettivamente a 7-8°C ed a 22°C. Tecniche di estrazione e separazione del DNA (PCR e DGGE) hanno rivelato la presenza di numerose specie microbiche in grado di condurre il processo di denitrificazione. Le due specie predominanti rivelate in entrambi i reattori sono state la *Dechloromonas denitrificans* e la *Hydrogenophaga caeni*.

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ABBREVIATIONS

AMD	Acid mine drainage
ANFO	Ammonium nitrate fuel oil
AOM	Anaerobic oxidation of methane
COD	Chemical oxygen demand
COD_{in}	Influent COD
COD_{out}	Effluent COD
CSTR	Completely stirred tank reactor
DOC	Dissolved organic carbon
DFFBR	Down-flow fluidized-bed reactor
DGGE	Denaturing gradient gel electrophoresis
DNRA	Dissimilatory nitrate reduction to ammonium
FBR	Fluidized-bed reactor
GAC	Granular activated carbon
GLR	Gas lift reactor
HB	Homoacetogenic bacteria
HPLC	High performance liquid chromatograph
HRT	Hydraulic retention time

IC	Ion chromatograph
IFBR	Inverse fluidized-bed reactor
MA	Methanogenic archaea
MBR	Membrane biological reactor
PBR	Packed bed reactor
PCL	Poly caprolactone
PCR	Polymerase chain reaction
PHB	Poly 3-hydroxybutyrate
RID	Refractive index detector
$SO_4^{2-}{}_{in}$	Influent sulfate concentration
$SO_4^{2-}{}_{out}$	Effluent sulfate concentration
SRB	Sulfate-reducing bacteria
SRP	Sulfate-reducing prokaryotes
SRT	Sludge retention time
TSS	Total suspended solids
UASB	Up-flow anaerobic sludge blanket
UFBR	Upflow fluidized-bed reactor
VSS	Volatile suspended solids

Chapter 1

Introduction

In the last decades, increasing attention of the scientific community has been given to the treatment of mining waters and to the polluting effects of various sulfur compounds and heavy metals. Anthropogenic activities may cause disturbances in the natural sulfur cycle leading to alteration of all the environmental components (water, air, soil and sediments) (Lens et al., 2002). Pollution by high sulfate concentration waste streams often results in the leaching of metals, especially in acid mine drainage (AMD) (Johnson and Hallberg, 2005). Metals such as Cd, Cr, Cu, Pb and Zn are toxic and non-biodegradable pollutants which tend to accumulate in the food chain and are absorbed by living organisms, including the human body causing serious health disorders (Roberts and Johnson, 1978; Zhuang et al., 2009). Besides, they also affect the aesthetic quality of potable water (Gray, 2008).

The exploitation of sulfide minerals results in the oxidation of iron and sulfur and the formation of waters characterized by low pH and high metal and sulfate concentrations (Foucher et al., 2001). However, mining industry is not only responsible for sulfate and heavy metal contamination of waters, but it often results in strong nitrogenous compounds discharge, e.g. ammonium and nitrate (Häyrynen et al., 2009). Nitrate is one of the most common contaminants into the aquatic environment worldwide, especially in groundwater (Power and Schepers, 1989; Korom et al., 1992). It usually originates from the large use of fertilizers in agriculture and uncontrolled industrial drain streams (Park and Yoo, 2009). The main source of nitrate in AMD originates from N-based blasting agents, such as ammonium nitrate fuel oil (ANFO), that remain partially undetonated (Forsberg and Åkerlund, 1999; Koren et al., 2000). Moreover, for gold extraction activities, cyanide is often used as lixiviant contributing to the release of nitrate in the rocks and ores (Zagury et al., 2004). Nitrate contamination of drinking and waste waters can lead to human health problems and eutrophication of the water bodies, respectively (Calderer et al., 2010).

Preventing the formation or the migration of AMD is generally considered to be the preferable option, although this is not feasible in many locations and, in such cases, it is necessary to collect, treat and discharge mine waters (Johnson and Hallberg, 2005). Traditionally, heavy metal- and sulfate-containing wastewaters can be treated using several technologies including adsorption, cementation, coagulation-flocculation, ion exchange, membrane separation or precipitation (Brooks, 1991; Kurniawan et al., 2006; Fu and Wang, 2011). Most industries treat these wastewaters by precipitating metals with hydroxide or carbonates and sulfate with $Ca(OH)_2$ essentially because of process simplicity and ease of process control (Veeken et al., 2003a). However, this method presents some drawbacks in terms of application and effectiveness as it usually results in the production of unstable sludge, which leads to a greater disposal expense (Tabak and Govind, 2003b; Esposito et al., 2006).

In recent years, the use of biological processes for treating AMD has gained increasing interest, mainly due to their ability to produce effluents suitable to be discharged into the environment (Janssen et al., 2001). When sulfate is present in the wastewater stream, biogenic sulfide produced by sulfate-reducing bacteria (SRB) is an important alternative sulfide source for metal precipitation. The removal mechanism is based on the fact that, under anaerobic conditions, SRB can oxidize simple organic compounds using sulfate as terminal electron acceptor which is reduced to sulfide (Dvorak et al., 1992; Lens et al., 2000). Removal of metals is due to the production of highly insoluble precipitates that react with the biogenic H_2S (Lewis and van Hille, 2006; Villa-Gòmez et al., 2012). Among the electron donors that SRB can use, lactate was used as carbon source/electron donor in the present study to develop a biological sulfate-reducing process. The stoichiometric reaction between lactate and sulfate is the following:

$3SO_4^{2-} + 2CH_3CHOHCOOH \rightarrow 3H_2S + 6HCO_3^-$ (1)

Lactate can be completely oxidized to HCO_3^-, as reported in the previous reaction, or partially oxidized to acetate (Oyekola et al., 2009) (equation 2):

$SO_4^{2-} + 2CH_3CHOHCOOH \rightarrow 2CH_3COOH + H_2S + 2HCO_3$ (2)

In this case an important organic residual pollution is possible due to the difficulty of removing acetate in a sulfidogenic bioreactor, as well as a not complete pH neutralization due to the lower alkalinity produced (Kaksonen et al., 2003b).

Normally, nitrate removal from wastewaters is conducted biologically, as in municipal wastewater treatment plants (Keller et al., 2002). Biological denitrification occurs mainly under anoxic conditions in the presence of heterotrophic bacteria using nitrate as electron acceptor (Dahab and Lee, 1988; Gayle et al., 1989). Mine waters notoriously lack of organic content and, thus, organic compounds have to be injected to promote the biological process as well as for sulfate reduction (Borden et al., 2012). Acetate, ethanol, glucose, methanol and methane have been all demonstrated to be very suitable for carrying out denitrification. Ethanol has been found to be the most effective in terms of denitrification rates, reaction completeness and microbial growth (Christensson et al., 1994; dos Santos et al., 2004). The reaction between ethanol and nitrate is expressed by the following equation (3):

$12NO_3^- + 5CH_3CH_2OH \rightarrow 6N_2 + 10CO_2 + 9H_2O + 12OH$ (3)

If the reaction develops completely, nitrate is totally converted to nitrogen gas N_2 that releases to the gas phase. Moreover, OH^- ions are produced neutralizing the eventual acidic pH of the solution. On the contrary, if nitrate is partially reduced, nitrite accumulates in solution as intermediate of the reaction (4):

$6NO_3^- + CH_3CH_2OH \rightarrow 6NO_2^- + 2CO_2 + 3H_2O$ (4)

Nitrite still represents nitrogen pollution and no alkalinity is produced to neutralize the initial pH.

Recently, the development and improvement of several bioreactor configurations led to effective removal of sulfate, nitrate, metals and acidity from mine waters either by immobilization of microbes on solid substrate or keeping microorganisms in suspension (Lens et al., 2002; Cohen 2006; Zaitsev et al., 2008). Fluidized-bed reactors (FBR), operated both in up-flow and down-flow modes, were chosen as bioreactor type in the present work. Classical FBR (up-flow) and inverse fluidized-bed reactors (IFBR – down-flow) are gaining increasing attention among all the different reactor designs due to their advantages and high efficiency in wastewater treatment (Iza, 1991; Green et al., 1994; Nicolella et al., 1997).

The present thesis aims at the possibility of removing metals, sulfate and nitrate from simulated mine waters. Firstly, the effect of sulfide concentration on the settling properties of metal precipitates as well as the contribution of other mechanisms for metal removal in sulfate-reducing bioreactors was investigated. Batch experiments following metal depletion kinetics were carried out with biofilm-coated polyethylene beads, sulfide at different concentrations, macronutrients and metals (Zn, Cu, Pb and Cd). X-ray absorption spectroscopy was also applied in order to study specifically the molecular structure of Zn-containing precipitates and complexes.

Secondly, biological sulfate-reducing and nitrate-removing processes were performed for the treatment of low-pH simulated mine waters using the fluidized-bed technology. In the scientific literature, many efforts have been made to study the sulfate reduction under acidic conditions with contradictory

results, whereas literature lacks studies focused on the effects of very low pH on denitrification. Therefore, two sulfate-reducing IFBRs and three nitrate-removing classical FBRs were operated for the enrichment of SRB and denitrifying bacterial cultures on lactate and ethanol, respectively. In particular, the sulfate-reducing application was directed to: 1) evaluate the sulfate-reducing process efficiencies under different COD/sulfate ratio conditions; 2) determine the optimal amount of organic matter to add to the solution; 3) test the reactors under sudden pH decreases in the feed solution.

Finally, the experimentation for nitrate removal, carried out both in continuous FBRs and several batch assays, aimed to: 1) study the effect of low temperature, low pH and HRT on the performance of biological denitrification of low-organic content simulated mine waters; 2) evaluate the best ethanol/nitrate ratio for achieving the highest nitrate removal efficiencies; 3) monitor the development of different microbial cultures enriched and maintained within the bioreactors.

Chapter 2

Literature review

2.1 AMD AND MINING WASTEWATERS

2.1.1 Sulfide minerals

Mining activities consist in the introduction of an oxidizing agent (oxygen or water) which leads to the oxidation of metal-based minerals, naturally present in a reduced state (Banks et al., 1997). A lot of economically important metals are present in nature combined with sulfur as metal sulfides (Table 1) (Woods, 2004). The general formula of metal sulfide is given by M_mS_n where M represents the metallic element and S sulfur (Klein and Hurlbut Jr., 1985). The most widespread mineral is pyrite either in metal sulfide and coal deposits. Other common sulfide minerals are sphalerite (ZnS), galena (PbS), arsenopyrite (FeAsS), chalcocite (Cu_2S), cobaltite (CoAsS) and millerite (NiS) (Woods, 2004).

Table 1 - Metals extracted from the main mineral groups (adapted from Woods, 2004)

Mineral group	Metals
Arsenides	As, Co, Ni
Oxides	Al, Fe, Mn, Mo, Sn
Silicates	Be, Li, U, Zn
Sulfides	Ag, Cd, Co, Cu, Hg, Mo, Ni, Pb, Zn

Most of metal sulfide minerals are of igneous and sedimentary origin (Jensen, 1989; Ehrlich, 2002). Particularly, sulfides in sedimentary rocks are thought to be of biogenic origin since most biogenic metal sulfides are associated with bacterial sulfate reduction in anaerobic environments, such as organic-rich sediments in seas or lakes (Shimazaki et al., 1985).

2.1.2 AMD formation

AMD is an outflow of acidic water from coal mines or metal mines. AMD is generated through a combination of chemical and biological processes by which metal sulfides are converted to sulfate and metal hydroxides when exposed to fresh water and oxygen (Tichy et al., 1998; Neculita et al., 2007). Moreover, acid mine drainage formation is further amplified when the reactions are catalyzed by aerobic bacteria such as *Acidithiobacillus ferrooxidans* (Robb, 1994; Brown et al., 2002). The mechanism for the production of AMD is presented in the following reactions (5-8), which describe the oxidation of pyrite ore as a typical example (Banks et al., 1997; Kaksonen and Puhakka, 2007):

$$2FeS_2 + 7O_2 + 6H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H_3O^+ \quad (5)$$

The oxidation of ferrous to ferric iron consumes protons:

$$4Fe^{2+} + 4H_3O^+ + O_2 \rightarrow 4Fe^{3+} + 6H_2O \quad (6)$$

Hydrolysis of ferric iron occurs subsequently which releases protons:

$$Fe^{3+} + 6H_2O \rightarrow Fe(OH)_3 + 3H_3O^+ \quad (7)$$

The overall sequence of reactions is acid-producing:

$$4FeS_2 + 30H_2O + 15O_2 \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 8H_3O^+ \quad (8)$$

Other sulfide minerals are oxidized in similar way as pyrite, releasing metals and sulfate in the same way. However, the oxidation of sulfides with generic formula MS does not release acid, e.g. sphalerite oxidation (Banks et al., 1997):

$$ZnS + 2O_2 \rightarrow Zn^{2+} + SO_4^{2-} \quad (9)$$

As shown in the previous reactions, the water pH progressively decreases resulting in further dissolution and mobilization of heavy metals from mine wastes (van Houten et al., 1994). Consequently, disposing AMD without an appropriate treatment leads to environmental contamination (Jong and Parry, 2003). Pollution control of AMD can be achieved by preventing AMD formation and/or collection and treatment of the AMD (Geldenhuis and Bell, 1998; Johnson, 2000).

AMD from abandoned mines is a major environmental issue in the United States and other countries wherever mining has been practiced on a large scale (Tabak et al., 2003a), since this drainage typically contains high concentrations of dissolved metals (Table 2) and more than 3 g/L sulfate (Tabak and Govind, 2003b). The formation of acidic wastewaters can even continue for tens or hundreds of years after mine closure (Béchard et al., 1994). AMD and mining water characteristics change according to the chemical soil composition the waters pass through. Figure 1 shows two pictures of Sotkamo mine, an open pit and underground Fe, Ni, Co and As mine in Central Finland. Table 2 gives some examples of the dissolved metal concentrations in Berkeley Pit AMD (USA) (Tabak et al., 2003a), in the Gromolo river in Libiola mine area (Italy) (Dinelli et al., 2001; Dinelli and Tateo, 2002), in Kennecott Copper mine AMD (USA) (Buisman et al., 1999) in Queen mine wastewater (USA) (Ashe et al., 2008) and in Leviatham mine wastewater (USA) (Tsukamoto et al., 2004).

Figure 1 - The open pit and underground mine of Sotkamo, Central Finland

Table 2 - Typical AMD and mining wastewater composition (the unit is mg/L)

Compound	Berkeley Pit AMD	Gromolo river	Kennecott Copper AMD	Queen mine wastewater	Leviatham mine water
Al^{3+}	293	230	2412	3950	-
Cu^{2+}	223	175	44	-	0.7
Mn^{2+}	223	9.15	200	1620	-
Fe^{2+}	514	775	512	2500	220
Zn^{2+}	630	35	82	930	0.9
Cd^{2+}	1.38	-	-	-	-
Ni^{2+}	2.14	8.5	-	-	0.5
As^{3+}	0.512	-	-	-	30
Co^{2+}	1.51	-	-	-	-
Cr^{3+}	-	1.23	-	-	-
Na^{+}	213	442	-	-	-
Mg^{2+}	-	1080	2640	2890	-
Ca^{2+}	-	333	-	-	-

Nutrients such as nitrogen and phosphorus can be present in mine waters (Johnson, 2000). Ammonium and nitrate are the dominant forms of nitrogen and considerable amounts of these compounds are essentially due to the use of N-based explosives in mining (Koren et al., 2000). Moreover, high salinity concentrations have been found in mine waters because of the intrusion of seawater in coastal mines (Banks et al., 1997). Finally, AMD formation can be also attributed to the oxidation of minerals in coal deposits. Coal usually contains both inorganic sulfur (mostly pyrite and sulfate) and organic sulfur (sulfide, sulfoxides and sulfones) in total concentration of 1-10 % (Johnson, 2000).

2.1.3 Environmental impact of wastewaters

Gray (1997) and Jarvis and Younger (2000) categorized the effects of AMD as chemical, physical, biological, ecological and socioeconomic impacts (Table 3)

Table 3 - Major impacts of AMD

Chemical	Physical	Biological	Ecological	Socioeconomic
Increase of acidity	Increase of turbidity	Respiration problem	Ecosystem modification	Aesthetic loss
Lixiviation of heavy metals	Decrease of light penetration	Reproduction problems	Decrease of primary production	Corrosion
Change of natural sulfur cycle	Decrease of oxygen diffusion	Acute and chronic toxicity	Bioaccumulation in food chain	Alteration of water supplies
Production of toxic hydrogen sulfide	Adsorption of metals on sediments	Death of sensitive species	Food chain modification	Health effects
		Migration		Decrease in catch of fish

The major natural impact areas are rivers, lakes, estuaries and coastal waters, but contamination can also regard groundwater resources and arable lands (Johnson, 2000). The acidity of AMD can cause direct toxic effects to the aquatic environments or influence indirectly by increasing the solubility of toxic metals and affecting the natural sulfur cycle (Lens et al., 2002). AMD-impacted water courses have been seen to limit the biodiversity in microorganisms and planktonic organisms if compared to non-polluted waters (De Nicola and Stapleton, 2002). The presence of iron precipitates affects the aesthetic quality of the water bodies and limits oxygen diffusion, increasing the turbidity of water streams and reducing light penetration and primary production (Johnson, 2000). The quality of the water supplies used for agricultural, industrial and recreational purposes is also strongly affected. Moreover, acidic waters can lead to serious damages to manmade constructions such as foundations, bridges and dams (Jarvis and Younger, 2000).

2.1.4 Abiotic AMD treatment

For lowering the impacts of mine waters to the natural environments, pollution control can be achieved by limiting AMD formation or suitably treating it. The techniques adopted for preventing or limiting AMD formation are known as source and migration control measures (Geldenhuis and Bell, 1998). Basically, these techniques rely on the reduction of oxidizing agents entry to the source of AMD and the restriction of the movement of contaminated waters (Geldenhuis and Bell, 1998). Exclusion of air or rainwater (Evangelou and Zhang, 1995; Perry et al., 1998), revegetation (Strock, 1998), immobilization of the soil contaminants (Jang et al., 1998) and the use of microbial inhibitors (Kleinmann, 1998; Seidel et al., 2000) are some of the techniques adopted for source and migration control.

In most cases, especially at operating mines, the only practical option is the treatment of the mining effluents (Geldenhuis and Bell, 1998). The traditional AMD treatment techniques are abiotic and include neutralization with alkaline substances, chemical metal precipitation and other alternative physico-chemical processes such as ion exchange and adsorption (Kurniawan et al., 2006). Most of the abiotic techniques are directed to metal removal and pH neutralization, whereas sulfate remains in solution (Tichy et al., 1998). Limestone ($CaCO_3$) is often used as alkaline substance to increase the water pH and to precipitate metals (Hedin et al., 1994; Cravotta and Trahan, 1999; Santos et al., 2004). The benefits of using limestone as alkalinity source are due to the cost and ease of conducting the neutralization process. The reactions between limestone and acidity is expressed in the following reactions (10-11) (Nairn et al., 1992; Gazea et al., 1996):

$$CaCO_3 + 2H_3O^+ \rightarrow Ca^{2+} + H_2CO_3 + H_2O \qquad (10)$$

$$CaCO_3 + H_2CO_3 \rightarrow Ca^{2+} + 2HCO_3^- \qquad (11)$$

This treatment is hardly effective when the influent contains appreciable amounts of ferrous iron and it is in contact with limestone in an oxidizing environment, as limestone is quickly coated with iron precipitates slowing down its dissolution and alkalinity production (Robb and Robinson, 1995; Gazea et al., 1996).

Metal removal can be also performed by using chemical precipitation (Peters et al., 1985; Veeken and Rulkens, 2003b). Classical metal precipitation is conducted by using alkaline substances, such as hydrated lime ($Ca(OH)_2$) and caustic soda (NaOH), that precipitate metals as hydroxides (Peters et al., 1985). The pH values where metal hydroxides show the minimum solubility results to be in the range 7.5-11 (Conner, 1990). Each metal has a different optimum pH

that can be automatically controlled (Veeken and Rulkens, 2003b). Despite this advantage, hydroxide precipitation has some drawbacks such as: *i)* high costs for the large amount of chemical used, *ii)* the inefficient removal of sulfate and *iii)* the production of high bulky sludge volumes that require further treatment and high disposal costs (Kurniawan et al., 2006; Aziz et al., 2008). Moreover, such a gelatinous sludge makes hardly suitable the hydroxide precipitation process for metal recovery (Tabak and Govind, 2003b; Esposito et al., 2006). In fact, since heavy metals are non-renewable resources, their recovery and reuse is as important as their removal from wastewaters for both economic and environmental reasons (Badmus et al., 2007).

Therefore, recently the stabilization of metals is preferred in the form of sulfide precipitates. Besides a better selective metal recovery, sulfide precipitation has been demonstrated to be superior over hydroxide precipitation as *i)* effluent concentrations are orders of magnitude lower (µg/l vs. mg/l), *ii)* high reaction rates result in low hydraulic retention times (HRT) and *iii)* metal sulfide sludge is more compact and exhibits better settling, thickening and dewatering characteristics than hydroxide sludge (Brooks, 1991; Hammack et al., 1993; Peters et al., 1993; Luther et al., 1996; Veeken et al., 2003a). The solubility products of most metal sulfides and hydroxides are reported in Table 4 (Dean, 1999). Finally, sulfide can be naturally produced in biosulfidogenic reactors, appreciably reducing the operational costs and promoting sulfate reduction as well (Hammack et al., 1993; Bhagat et al., 2004; Esposito et al., 2006).

Table 4 - Solubility products of metal hydroxides and sulfides at temperatures between 18 and 25°C

Metal	**Solubility product (mol/L)**	
	OH^-	**S^{2-}**
Al^{3+}	1.3×10^{-33}	2.0×10^{-7}
Ag^{+}	2.0×10^{-8}	6.3×10^{-50}
As^{3+}	-	2.1×10^{-22}

Ca^{2+}	5.5×10^{-6}	-
Cd^{2+}	7.2×10^{-15}	8.0×10^{-27}
Co^{2+}	5.9×10^{-15}	2.0×10^{-25}
Cr^{3+}	6.3×10^{-31}	-
Cu^{2+}	2.2×10^{-20}	6.3×10^{-36}
Fe^{2+}	4.9×10^{-17}	6.3×10^{-18}
Fe^{3+}	2.8×10^{-39}	-
Hg^{2+}	2.0×10^{-24}	1.0×10^{-47}
Mg^{2+}	5.6×10^{-12}	-
Mn^{2+}	1.9×10^{-13}	2.5×10^{-13}
Ni^{2+}	5.5×10^{-16}	2.0×10^{-26}
Pb^{2+}	1.4×10^{-15}	8.0×10^{-28}
Zn^{2+}	3.0×10^{-17}	1.6×10^{-24}

Other physico-chemical technologies such as ion exchange, membrane filtration and adsorption are also available to treat AMD waters even if they are expensive and not commonly used (Prasad et al., 1999; Cohen, 2006). Ion exchange permits to effectively treat inorganic effluent with a metal concentration of less than 10mg/L, or in range of 10-100 mg/L. A reversible interchange of ions between the solid and liquid phases occurs by using insoluble resins that remove ions from an electrolytic solution and release other ions in a chemically equivalent amount without any structural change of the resin (Kurniawan et al., 2006). When ion exchange resins are saturated, they must be regenerated with an acid solution to remove metal ions from the resin bed. In this way, heavy metals can be even recovered in a more concentrated form by elution with appropriate reagent (Lanouette, 1977; Scheeren et al., 1991).

In membrane extraction, heavy metals are removed by using membranes which separate liquid and solid phases. Metals are leached in an organic solvent that is regenerated in a stripping module from where it is recycled back to the extraction module (Peters et al., 1985). Depending on the size of the particle that can be retained, various types of membrane filtration such as ultrafiltration, nanofiltration and reverse osmosis can be employed for heavy metal removal.

However, most of these techniques are prone to fouling and the regeneration can be very frequent (Kurniawan et al., 2006).

Adsorption is used as metal removal technique as well and activated carbon is the most widely material employed. It has been demonstrated that activated carbon is capable to adsorb metals such as hexavalent chromium, mercury and metal compounds complexed in organic forms (Lanouette, 1977). The size of the pores, solution pH and concentration of the target molecule in the liquid phase affect the adsorption capacity of activated carbon. As for ion exchange and membrane filtration techniques, activated carbon beds have to be generated. Therefore, the treatment process is conducted in a semi-continuous way unless more than one unit are used simultaneously (Scheeren et al., 1991).

2.2 SULFATE REDUCTION BIOTECHNOLOGY

In recent years the use of biological processes for treating heavy metal-bearing wastewaters has gained increasing interest as an alternative way, mainly because of their ability to produce effluents which are suitable to be discharged into the environment (Goncalves et al., 2007). Bacterial sulfate reduction is considered as an important bio-process for removing sulfate and metals from metal-mine drainage by means of sulfate-reducing bacteria (SRB) (Tuttle et al., 1969; Wakao et al., 1979; Herlihy and Mills, 1985; Hedin et al., 1989). The mechanism of removal is based on the fact that SRB can oxidize simple organic and inorganic compounds by using sulfate as terminal electron acceptor in anaerobic respiration (Barton and Tomei, 1995). Sulfate is reduced to sulfide that is used to precipitate heavy metal from solution as metal sulfide (Cabrera et al., 2006):

$$H_2S + M^{2+} + 2H_2O \rightarrow MS(s) + 2H_3O^{+} \qquad (12)$$

where M stands for the general metal.

2.2.1 Microbiology of sulfate-reducing bacteria

Sulfate-reducing prokaryotes (SRP) are a heterogeneous group of bacteria and archaea capable of using sulfate as electron acceptor and reducing it to sulfide (Castro et al. 2000; Garrity et al., 2003). SRP consist of 220 species in 60 genera (Barton and Fauque, 2009) In literature, SRP have been classified according to different properties, including cell shape, electron transfer proteins, guanine cytosine content of DNA, optimal growth temperature and capability to oxidize acetate (Widdel, 1988; Akagi, 1995; Chen et al., 1995).

SRP are divided in six different classes: *Archaeoglobi*, *Thermoprotei*, *Thermodesulfobacteria*, *Nitrospira*, *δ-Proteobacteria* and *Clostridia* (Garrity et al., 2003). The majority of the described species of SRP are bacteria, therefore SRP are simply named SRB most of times (Rabus et al., 2006). Depending on the growth temperature, most of SRB identified are mesophilic, but thermophilic, hyperthermophilic and phychrophilic species have also been described (Table 5) (Knoblauch et al., 1999; Jeanthon et al., 2002).

Table 5 - The optimal growth temperature (T_{opt}) of selected SRP and their ability to oxidize acetate

SRP species	**T_{opt} (°C)**	**Acetate oxidation**	**References**
Gram-negative mesophilic			
Desulfobulbus	28-39	-	Widdel and Pfenning (1984)
Desulfomicrobium	25-40	-	Castro et al. (2000)
Desulfovibrio	25-40	-	Widdel and Pfenning (1984)
Desulfobacter	28-32	+	Widdel and Pfenning (1984)
Desulfobacterium	20-35	+	Castro et al. (2000)
Desulfococcus	15-36	+	Widdel and Pfenning (1984)
Desulfomonile	37	+	Castro et al. (2000)
Desulfonema	28-32	+	Castro et al. (2000)
Desulfosarcina	28-33	+	Widdel and Pfenning (1984)
Gram-positive sporulating			
Desulfotomaculum	25-65	+/-	Castro et al. (2000)
Psychrotolerant mesophilic			
Desulforhopalus vacuolatus	18-19	-	Isaksen and Teske (1996)
Desulfofrigus fragile	18	-	Knoblauch et al. (1999)
Desulfotalea arctica	18	-	Knoblauch et al. (1999)
Desulfovibrio ferrireducens	23	-	Vandieken et al. (2006)
Desulfovibrio frigidus	20-23	-	Vandieken et al. (2006)
Desulfobacter hydrogenophilus	29-32	+	Widdel (1987)

Desulfobacter psychrotolerans	20	-	Tarpgaard et al. (2005)
Desulfobacterium autotrophicum	25-28	+	Rabus et al. (2002)
Psychrophilic			
Desulfofrigus oceanense	10	+	Knoblauch et al. (1999)
Desulfofaba gelida	7	-	Knoblauch et al. (1999)
Desulfotalea psychrophila	10	-	Knoblauch et al. (1999)
Thermophilic			
Thermodesulfobacterium	65-70	-	Castro et al. (2000)
Hyperthermophilic			
Archaeoglobus	64-92	-	Castro et al. (2000)

Another important classification criterion for SRB is the ability of oxidizing acetate (Table 5). Brock et al. (1994) divided the genera of heterotrophic sulfate-reducing bacteria into two main physiological subgroups. The genera in the first group (*Desulfovibrio, Desulfomonas, Desulfotomaculum and Desulfobulbus*) utilize lactate, pyruvate, ethanol and certain fatty acids as carbon source but are not capable to oxidize acetate to CO_2. The genera in the second group (*Desulfobacter, Desulfococcus, Desulfosarcina and Desulfonema*) are specialized in the oxidation of short chain fatty acids, especially acetate. Incomplete oxidation is due to the absence of a mechanism for Acetyl-CoA (coenzyme A) oxidation (Widdel and Hansen, 1992). The reason for this is still unknown and more efforts should be done on the pathway used by SRB to produce and degrade acetate (Rabus et al., 2006).

Sulfate reduction with an electron donor proceeds according to the following equation (Postgate, 1984; Widdel, 1988; Dvorak et al., 1992):

$$SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2HCO_3^- \qquad (13)$$

All the sulfate reduction reactions take place in the cytoplasm, thus the cells must have an efficient sulfate transport system (Cypionka, 1989). The two products of the reactions are hydrogen sulfide and bicarbonate ions and they are released out of the cells again. On the contrary, the intermediate sulfur compounds are not excreted (Rabus et al., 2006).

The function of bicarbonate ions is very important. In low-pH wastewaters, they react with protons to form CO_2 and water and remove acidity from solution as CO_2 gas:

$$HCO_3^- + H_3O^+ \rightarrow CO_2\,(g) + 2H_2O \qquad (14)$$

In this way, the pH of the solution is neutralized promoting better environmental conditions for SRB (Dvorak et al., 1992).

Some SRB are autotrophic microorganisms capable of growing with CO_2 or CO as a sole carbon source (Klemps et al., 1985). In this case, molecular hydrogen is used as energy source and electron donor (Widdel, 1988; van Houten et al., 1994; Esposito et al., 2003):

$$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O \qquad (15)$$

2.2.2 Electron donors

Generally acid mine drainage contains very low concentrations of dissolved organic carbon (Kolmert and Johnson, 2001). For this reason, the additional organic carbon source to be added as electron donor determines the overall costs of the sulfate reduction bio-process (Gilbert et al., 2004; Zagury et al., 2006; Buisman et al., 2007). The choice of the substrate is based on different criteria: *i)* the ability of SRB to utilize the organic substrate, *ii)* the sulfate load to be

reduced and the cost of the substrate per unit of H_2S produced, *iii)* the availability in sufficient quantities and *iv)* the remaining pollution load from the incompletely degraded substrate (van Houten et al., 1994; Dries et al., 1998; Dijkman et al., 1999). In Table 6 the main organic electron donors used for sulfate reduction are listed with their respective advantages and shortcomings (Kaksonen and Puhakka, 2007; Liamleam and Annachhatre, 2007).

Table 6 - Carbon sources used for biological sulfate reduction

Carbon source	Advantages (+) / Shortcomings (-)	References
Formate	(+) Most of SRB that use hydrogen are able to oxidize formate as sole source of carbon	Widdel (1988)
	(-) Methanogens can predominate on SRB at thermophilic conditions	Vallero et al. (2004)
Methanol	(+) Cost effective	Glombitza (2001)
	(+) Predominance of SRB at high temperatures	Vallero et al. (2003)
	(-) Very few SRB species oxidize methanol	Widdel (1988)
	(-) Slow SRB growth at mesophilic conditions	Weijma and Stams (2001)
Ethanol	(+) High sulfate conversion efficiencies	Kaksonen et al. (2003b)
	(-) Accumulation of acetate as intermediate	Kaksonen et al. (2004b); Gallegos-Garcia et al. (2008)
Lactate	(+) High alkalinity production and biomass growth yields	Kaksonen et al. (2004a)
	(-) High cost	Nagpal et al. (2000b)
	(-) Accumulation of acetate as intermediate	Oyekola et al. (2009)
Acetate	(-) Methanogens generally outcompete SRB	Yoda et al. (1987)
	(-) Difficult to oxidize even with acetate-enriched cultures	Sahinkaya et al. (2007b)

	(-) Main responsible of organic rest pollution	Lens et al. (1998)
Fatty acids mixture	(+) Propionate is mainly oxidized by SRB at high sulfate concentrations	Visser et al. (1993)
	(-) Competition between methanogens and SRB even at high sulfate concentrations	Visser et al. (1993); Celis-Garcia et al. (2007)
	(-) Production of acetate as intermediate	Celis-Garcia et al. (2007)
Glucose and fructose	(+) Easily degraded and production of hydrogen as interspecies	(Klemps, 1985)
	(-) Production of volatile fatty acids as intermediates: competition between SRB and methanogens and decrease of pH	White and Gadd (1996b)
Aromatic hydrocarbons	(+) Degradation of dangerous substances such as phenols and benzene compounds	Harms et al. (1999); Lin and Lee (2001)
	(-) Low free energy change	Widdel (1988)
Molasses	(+) Ready availability and low costs	Maree and Strydom (1987)
	(-) Presence of non-biodegradable content	Annachhatre and Suktrakoolvait (2001)
Organic waste reactive mixtures	(+) High sulfate reduction rate because of high carbon content	Waybrant et al. (1998; 2002); Cocos et al. (2002)
	(+) Cost effective	Prasad et al. (1999)
	(-) High organic rest pollution	Glombitza (2001)
	(-) Synergism with other microbial groups	Kuyucak and St-Germain (1994)

SRB cannot directly oxidize complex organic compounds such as carbohydrates, proteins and lipids (Postgate, 1984). When these organic carbon sources are provided, synergism among different groups of microorganisms (such as fermentative bacteria and acidogens) is essential to guarantee the production of alcohols, H_2 and short-chain volatile fatty acids that SRB can use as substrates (Figure 2) (Tuttle et al, 1969; Kuyucak and St-Germain, 1994).

Heterotrophic SRB use the easily degradable fraction of organic matter such as low molecular weight compounds with simple structures, e.g. methanol, ethanol, propionate, butyrate, acetate, lactate and methane (Dvorak et al., 1992; Nagpal et al., 2000a; Tsukamoto et al., 2004; Meulepas et al., 2009). Autotrophic SRB applications rely on the use of hydrogen (van Houten et al., 1994; Esposito et al., 2003) or synthesis gas (van Houten et al., 1996) as electron donors, using CO_2 or CO as carbon source.

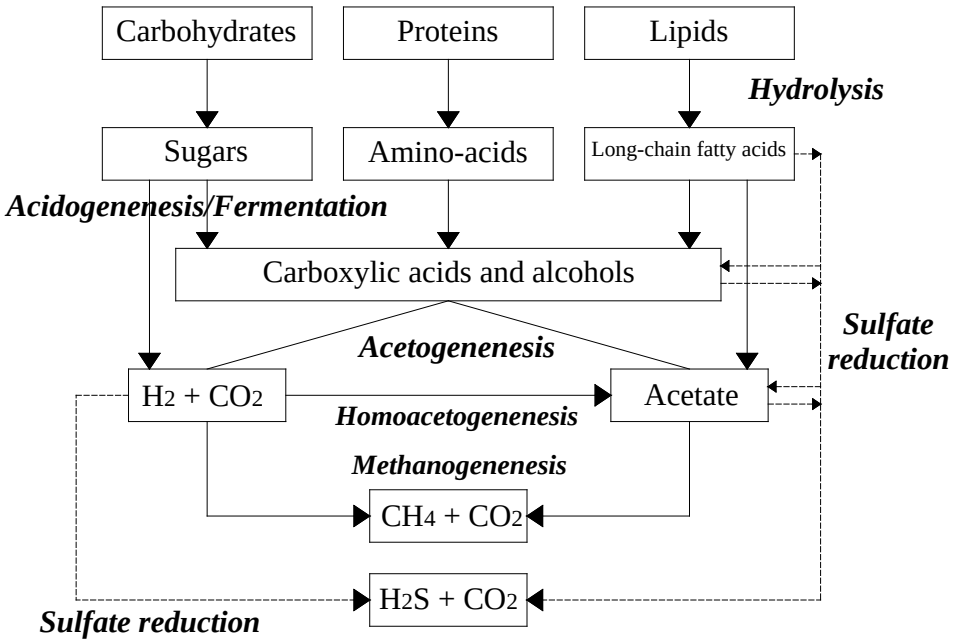

Figure 2 - Anaerobic digestion steps of organic matter with sulfate

Methanol has been largely used for sulfate reduction applications essentially for its availability and cost effectiveness (Glombitza, 2001; Weijma et al., 2003). However, under mesophilic conditions the growth of sulfate reducers on methanol is slow, with doubling times of one day or more (Weijma and Stams, 2001). In contrast, thermophilic conditions can favor sulfate reduction over

methanogenesis or homoacetogenesis when methanol is supplied as electron donor (Zinder et al., 1984; Weijma et al., 2000).

Ethanol is an attractive electron donor for sulfate reduction. Sulfate conversion efficiencies higher than 80% have been reached in many applications using ethanol as electron donor with high mass transfers (de Smul et al., 1997; Kaksonen et al., 2003b). Moreover, ethanol has been found to be oxidized completely by several cultures of *Desulfovibrio desulfuricans* and *Desulfobacter postgatei* (Nagpal et al., 2000a). However, SRB cultures enriched on ethanol show lower biomass growth yields and, in most of cases, ethanol is partially degraded to acetate not producing any alkalinity (equation 16):

$$2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H_2S + 2H_2O \qquad (16)$$

Propionate and butyrate are common fermentation products in an anaerobic processes such as sulfate reduction (Speece et al., 1996). Different sulfate-reducing species have been shown to degrade propionate and butyrate either completely and partially to acetate (Widdel, 1988; Harada et al., 1994). Nevertheless, the use of H_2 as further source of energy is essential for SRB to oxidize propionate and butyrate (Thauer et al., 1977). Generally, propionate is preferred to butyrate since incomplete propionate oxidation produces alkalinity for pH neutralization (Thauer et al., 1977).

Acetate oxidation is important in AMD treatment because acetate represents a rest pollution of the supplied electron donor (Kaksonen et al., 2004b). Acetate can be either excreted from the cells, if the bacterium does not have the necessary enzyme to degrade it, and used as electron donor and carbon source in the sulfate reduction process (Celis-Garcia et al., 2007). Nonetheless, very few SRB species can use acetate as sole carbon source (e.g. *Desulfotomaculum acetoxidans*) (Koschorreck et al., 2004) and some incomplete oxidizers are able

to use acetate if H_2 is used as electron donor (e.g. *Desulfotomaculum alkaliphilum)* (Pikuta et al., 2000; Koschorreck et al., 2004).

Lactate is a superior electron donor compared to others such as ethanol, acetate or propionate in terms of energy and biomass produced (Nagpal et al, 2000b) and it is also superior compared to ethanol in terms of moles of bicarbonates produced, thus neutralizing the effluent acidity in a better way (Kaksonen et al., 2004a). Two important drawbacks are that 1) lactate is expensive and 2) only particular species of sulfate-reducing bacteria (*Desulfotomaculum*) are able to oxidize lactate to CO_2, whereas others (*Desulfovibrio desulfuricans*) oxidize lactate to acetate (Koschorreck et al., 2004). Another shortcoming associated with the use of lactate is that it exists predominantly as undissociated molecule in acidic AMD waters, which could be inhibitory or lethal to SRB (Johnson et al., 2002).

Sulfate reduction coupled to anaerobic oxidation of methane (AOM) is an appealing research perspective and forms an important process in the global sulfur and carbon cycles (Valentine and Reeburgh, 2008; Meulepas et al., 2009). Methane is largely available since it is present in natural gas and biogas. It is known that sulfate reduction via AOM is conducted by at least two different groups of marine archaea (Nauhaus et al., 2002). However, the growth of these microorganisms is the most important limitation to overcome since the doubling time of the archaea has been estimated around 7 months (Nauhaus et al., 2007). Finally, the low optimum temperature and the high salt requirement limit the operational window of the process (Meulepas et al., 2009).

SRB can be autotrophic and use hydrogen as electron donor and further source of energy. Hydrogen is the energetically most favorable electron donor for SRB due to the high free energy change of sulfidogenic oxidation (ΔG_0 = -152 kJ/mol) (van Houten et al., 1994). A major problem of the hydrogen fed sulfate-

reducing applications is the competition among SRB and other H2-utilizing microorganisms such as methanogenic archaea and homoacetogenic bacteria (Weijma et al., 2002). When hydrogen is used as electron donor, CO_2 or acetate must be also added as carbon source for SRB (Widdel, 1988; van Houten et al., 1994). Hydrogen fed to sulfate-reducing bioreactors is commonly produced by reforming natural gas, requiring extra money and a separate unit process (Hammack and Dijkman, 1999). For lowering the capital costs, sulfate reduction can be also performed using synthesis gas (a mixture of H_2, CO_2 and CO) even if with lower efficiencies (van Houten et al., 1996)

The potential of the less expensive organic carbon sources, such as waste materials from agricultural and food processing industry, has been assessed to sustain sulfate reducing activity (Neculita et al., 2007). The use of these solid substrates has been particularly used for packed bed reactors since the substrates also function as a bacterial support. Several studies have reported that these complex substrates (such as alfalfa, hay, bales, straw, mushroom compost, municipal compost, animal manure, granular and sewage sludge, cellulose, wood, paper products, whey, compost) alone do not promote sulfate reduction significantly, whereas reactive mixtures containing more than one organic carbon source promote higher SRB activity (Christensen et al., 1996; Waybrant et al., 1998, 2002; Cocos et al., 2002; Zagury et al., 2006). The feasibility to sustain biological sulfate reduction through the utilization of activated sludge and digested sludge has also been studied (Prasad et al., 1999): the former was found to be the most suitable of these materials. For mine sites located near municipalities, it may be possible to link mine water treatment with the treatment of sewage sludge. Hammack et al. (1994) proved that a sulfate reducing treatment process could be coupled to a sewage treatment system who served to a population near to a pit water containing Zn and Cu.

2.2.3 Factors affecting the activity of SRB

2.2.3.1 Metal and sulfide toxicity

Heavy metals can be stimulatory for SRB activity at low concentrations and, at the same time, inhibitory or even lethal at high concentrations (Poulson et al., 1997; Sani et al., 2003). The toxicity of metals is due to their capacity to deactivate enzymes and to denature proteins (Cabrera et al., 2006). It depends on many factors such as biomass quantity, pH, temperature and initial metal concentration (Hao, 2000). The effect of metals on SRB can be in terms of *i)* inhibition of the bacterial growth, *ii)* extension of the lag phase in sulfide production, *iii)* decrease of sulfate reduction efficiency or *iv)* death of the bacteria (Table 7). In the case of biofilms, metal sulfide precipitation can form a barrier and can thus completely stop sulfate-reducing activity (Utgikar et al., 2003; Cabrera et al., 2006).

Table 7 - Toxic metal concentrations completely inhibiting sulfate-reducing activity

Heavy metal	Conc. (mg/L)	SRB species	References
Cd	6	SRB in Los Angeles County sewerage	Morton et al. (1991)
	> 22.4	SRB mixed and pure cultures	White and Gadd (1998)
	112	SRB mixture in manure sludge	Ueki et al. (1991)
Cr (III)	15	*Desulfovibrio vulgaris*	Cabrera et al. (2006)
Cu	1.9	*Desulfovibrio desulfuricans G20*	Sani et al. (2001)
	12	Mixed culture acetate-utilizing SRB	Utgikar et al. (2001)
	> 22.4	SRB mixed and pure cultures	White and Gadd (2000)
Ni	10	*Desulfovibrio desulfuricans*	Poulson et al. (1997)
	59	SRB mixture in manure sludge	Ueki et al. (1991)

Pb	2.1	*Desulfovibrio desulfuricans G20*	Sani et al. (2001)
	25	SRB in Los Angeles County sewerage	Morton et al. (1991)
Zn	13	*Desulfovibrio desulfuricans*	Poulson et al. (1997)
	20	Mixed culture acetate-utilizing SRB	Utgikar et al. (2001)
	20	*Desulfovibrio vulgaris* and *Desulfovibrio sp.*	Cabrera et al. (2006)
	65	SRB mixture in manure sludge	Ueki et al. (1991)

The presence of two or more different metals in a solution can have synergetic effects that cause an appreciably higher toxicity than expected on the basis of additive individual metal toxicity (Utgikar et al., 2004). Ueki et al. (1991) found that zinc completely inhibited SRB activity at 60 mg/l adding cattle waste to the acid mine water. Sani et al. (2001) assessed that only 6 µM of copper decrease the SRB maximum specific growth rate by 25% and, if this concentration increases to 30 µM, Cu (II) completely inhibits SRB growth. SRB growth begins only after a long lag time (e.g., 312 h) with 10 mM Pb (II) (Sani et al., 2003).

Inorganic cations (e.g. iron, calcium and magnesium) can have a positive effect on SRB inhibition by heavy metals: ions such as iron, calcium and magnesium compete with heavy metals on the anionic sites on cell surfaces reducing their toxicity (Collins and Stotzky, 1989).

Sulfate reduction results in the production of sulfide, which is inhibitory to different anaerobic trophic groups. Nevertheless, the presence of small quantities of sulfide in anaerobic reactors is advantageous since it constitutes an important sulfur source for some bacterial species such as the methanogens

(Daniels et al., 1986) and decreases the bioavailability of some toxic metals by the production of insoluble metal sulfides (Mizuno et al., 1994). The inhibitory effect of sulfide on SRB is direct, because of its intrinsic toxicity, and associated to the formation of insoluble metal sulfides with essential trace elements for microorganisms (Postgate, 1979; Reis et al., 1992). The toxic form of sulfide is H_2S because it passes through the cell membrane and inhibits the metabolic enzymes of the cells (Oleszkiewicz et al., 1989; O'Flaherty et al., 1998). H_2S concentrations are related to the temperature, the pH and its solubility in water (Figure 3):

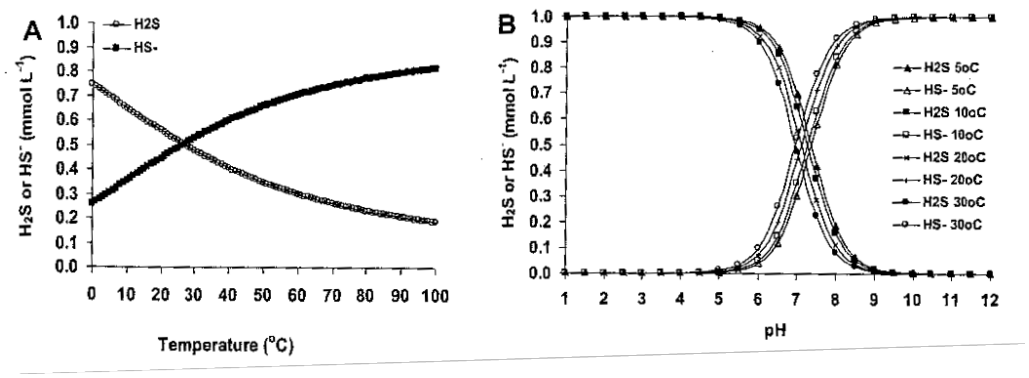

Figure 3 - The relative concentrations of H_2S and HS^- at temperatures 0-100°C (A) and at pH 1-12 at 5, 10, 15 and 20°C (B)

The pK_a of sulfide is 7.28 at 9°C, 6.99 at 25°C and 6.68 at 55°C (Kawazuishi and Prausniz, 1987; Amend and Shock, 2001). The proportion of the H_2S form increases as temperature decreases, and toxic H_2S concentration is reached at lower total dissolved sulfide concentration. Similarly, as the pH decreases the H_2S percentage increases. At pH 7, 30% of the sulfide is in the undissociated form. At pH 6, the percentage increases to 80% and, at pH 5 and 4, almost all the sulfide is present as H_2S. On the contrary, H_2S has a low solubility in water and it easily tends to strip by the biogas produced, decreasing its concentration

in the liquid phase. Not all the studies in scientific literature on the toxic effects of sulfide to SRB report similar results. For instance, Greben et al. (2005) observed an increasing sulfate reduction efficiency at sulfide concentrations as high as 1424 mg/L. On the contrary, Visser et al. (1993) showed that the process failed even at sulfide concentration as low as 50 mg/L. Several parameters affect the sensitivity of SRB to sulfide toxicity as reported in Table 8:

Table 8 - H_2S concentration values at which a 50% inhibition of SRB growth was observed

Biomass	**Substrate**	**T (°C)**	**pH**	**H_2S (mg/L)**	**Reference**
Granular non-sulfate adapted	Ethanol	37	6.8	294	O'Flaherty et al. (1998)
Granular sulfate adapted	H_2/CO_2	37	6.8	256	O'Flaherty et al. (1998)
Granular sludge	Acetate	30	7.3	171	Visser (1993)
Biofilm	Acetate/Ethanol	35	7	1300	Isa et al. (1986a; 1986b)
Biofilm	Acetate/Propionate	55	7	465	Celis-Garcia et al. (2004)
Digester sludge	Sugar/Ethanol	20-25	7.5	270-810	Greben et al. (2005)
Suspended sludge	H_2	35	7	380	Yamaguchi et al. (1999)
Desulfovibrio desulfuricans	Lactate	35	7	270	Okabe et al. (1992)
Desulfovibrio acetoxidans	Acetate	37	6.8	263	O'Flaherty et al. (1998)
Desulfovibrio vulgaris	Acetate	30	6.8	315	O'Flaherty et al. (1998)

2.2.3.2 Organic acids

Organic compounds can have toxic effects on SRB activity. Especially in low-pH bioreactors, the potential toxicity of the organic substrate needs to be considered because organic acids are predominantly in their non-ionized form (Reis et al., 1990). Undissociated acids enter the bacterial cell, acidify the cytoplasm and lead to bacterial death at high concentrations (Kimura et al., 2006). Acetic, propionic and butyric acids were reported to inhibit the growth of several microbial species (van den Heuvel et al., 1988). An acetic acid concentration higher than 2 mM or organic acids concentrations greater than 5 mM were found to completely inhibit SRB activity (Gyure et al., 1990). Lactic acid was also shown to affect sulfate reduction yields, either at acidic pH (Papirio et al., 2012a) and neutral pH (Reis et al., 1990). For these reasons, non-acid substrates yielding fermentation products as alcohols need to be taken into consideration as possible electron donors for sulfate reduction (Johnson et al., 2006; Kimura et al., 2006).

2.2.3.3 pH and salinity

Most engineering applications utilizing SRB have been carried out at neutral pH because of faster microbial growth and activity (Widdel, 1988; Hao et al., 1996; Willow and Cohen, 2003), but acid tolerant SRB have been shown to reduce sulfate even at pH as low as 3 (Kimura et al., 2006; Koschorreck et al., 2003) The toxic effect of low pH is due to acidification of cytoplasm, which inhibits the formation of a proton motive force (Thauer et al., 1977). Moreover, as said previously, low pH promotes the accumulation of undissociated VFA and sulfide that cause a similar effect to the cells. Since AMD wastewaters are strongly acidic (pH < 3), and such low pH normally affects microbial

metabolism, sulfate-reducing bioreactors apply different solutions (e.g. separated precipitation or water recirculation) to avoid direct contact between AMD and microorganisms. In this way, sulfidogenic bio-reactors have been inoculated with acidotolerant bacteria (Elliott et al., 1998; Sen and Johnson, 1999; Kolmert and Johnson, 2001) and successfully operated at pH 4-6 (Lopes, 2007; Bijmans et al., 2008a; 2008b).

Generally, high salinity has been shown to inhibit the operation of sulfidogenic bioreactors (Vallero et al., 2003). Nevertheless, SRB species from hypersaline lakes and environments have been found to tolerate salinity levels as high as 340 g/L (Ollivier et al., 1991). Using an ethanol/acetate- fed membrane bioreactor, sulfate reduction has been shown to be very efficient (Vallero et al., 2005).

2.2.3.4 Temperature

Most of the sulfate-reducing applications have been run at room temperature or under mesophilic conditions (25-45°C) (Madigan et al., 2000). Each sulfate-reducing species is classified according to its optimal temperature and temperature range at which the growth is the highest (Table 5). Generally, the growth and the conversion rates are higher at elevated temperature, but the energy needed to heat a bioreactor contributes to increase the costs. Therefore, it is more convenient to operate a bioprocess at a temperature close to the temperature of the sulfate-containing stream (Zinder et al., 1984).

At low temperatures, the kinetics of chemical and biological reactions sensibly slows down. The effects of temperature decrease and cold shocks are: *i)* decrease of catalysis and transport rates values; *ii)* inhibition of protein synthesis; *iii)* damage to the cell structure because of the formation of ice crystals at subzero temperature conditions (Cavicchioli et al., 2000). However,

several species have been found to immediately respond and acclimatize to quick temperature decrease (Bakermans et al., 2007), even if it results in a decrease of the enzymatic activity (Feller and Gerday, 2003)

2.2.3.5 Oxygen

Exposure to oxygen inhibits SRB metabolism, although the inhibition is reversible (Nagpal et al., 2000b). Some SRB species (such as *Desulfovibrio aerotolerans*) are capable to tolerate low levels of oxygen because they can oxidize extracellular polyglucose (Mogensen et al., 2005) or transform HS^- to partially oxidized species like $S_2O_3^{2-}$, which is reduced back to sulfide once anaerobic conditions are reinstalled (Wall et al., 1990).

2.2.4 SRB in engineered environments

Besides sulfate removal and metal precipitation/recovery in mining waters, the sulfate reduction bioprocess has been also applied to the treatment of tannery (Shin et al., 1996; Boshoff et al., 2004), textile (Albuquerque et al., 2005) , food production and brewery (Rodriguez-Martinez et al., 2005) and paper mill wastewaters (Thompson et al., 2001, Janssen et al., 2009). Moreover, biological SO_2 removal from flue gases has been shown to be feasible (Buisman et al., 2007). As a consequence of the increasing interest in applying biological processes for sulfate and metal containing-wastewater treatment, many different bioreactor designs have been developed (Speece, 1983; Hulshoff Pol et al., 2001; Lens et al., 2002; Kaksonen and Puhakka, 2007).

2.2.4.1 Reactor configurations

In the present work, an innovative and effective reactor configuration (the "fluidized-bed reactor") has been used either for sulfate and nitrate removal in acidic simulated mine waters. Other bioreactor designs used in previous studies for AMD treatment are:

- continuously stirred tank (CSTR) reactors (Barnes et al, 1991);
- packed-bed (PBR) reactors (Maree and Strydom, 1987; El Bayoumy et al., 1999; Jong and Parry, 2003);
- gas-lift (GLR) reactors (van Houten et al., 1994; Esposito et al., 2003; Bijmans et al., 2009);
- up-flow anaerobic sludge blanket (UASB) reactors (de Lima et al., 1996; Vallero et al., 2003; 2004);
- membrane biological (MBR) reactors (Chuichulcherm et al., 2001; Mack et al., 2004; Vallero et al., 2005).

Figure 4 gives a schematic representation of the different reactor configurations, whereas Table 9 overviews benefits and drawbacks.

Table 9 - Benefits and drawbacks of different bioreactor configuration operated for biological sulfate reduction

Bioreactor type	Benefits (+) / Drawbacks (-)	References
CSTR	(+) Consistency and reliability	Barnes et al. (1991)
	(-) High SRT result in high reactor volumes	Barnes et al. (1991)
	(-) Frequent active biomass washout	Lens et al. (2003)
PBR	(+) High SRT result in mower reactor volumes than CSTRs	Barnes et al. (1991)
	(+) Possibility to be operated both in up-flow and down-flow modalities	Jong and Parry (2003); Zaluski (2003)
	(-) Frequent clogging	Anderson et al. (1990)

	(-) High pressure for pumping the flow	Anderson et al. (1990)
GLR	(+) High mass transfer of the substrates into the bacterial agglomerates	Dijkman and Buisman (1999)
	(+) Very good mixing	Dijkman and Buisman (1999)
	(+) High rate biological kinetics if H_2 is used as electron donor	Van Houten et al. (1994; 1997)
	(-) High pressure needed for pumping the gaseous substrates inside the reactor	Lens et al. (2002)
UASB	(+) Biomass good settling capability	Lettinga et al., (1980)
	(+) No clogging	Omil et al. (1996)
	(+) No carrier material if compared to PBR	Speece (1983)
	(-) Possibility of biomass washout	Vallero et al. (2003)
	(-) High susceptibility to the influent characteristics	Jhung and Choi (1995)
MBR	(+) No need for sedimentation basin	Mack et al. (2004)
	(+) High biomass retention result in high substrate degradation rates	Mack et al. (2004)
	(+) Possibility to prevent direct contact between metals and SRB in a single basin	Chuichulcherm et al. (2001); Manconi and Lens (2009)
	(-) High cost to overcome the trans-membrane pressure	Fedorovich (2000)
	(-) Periodic backwash because of the deposition of aggregates on the membrane surface	Tabak and Govind (2003b); Vallero et al. (2005)

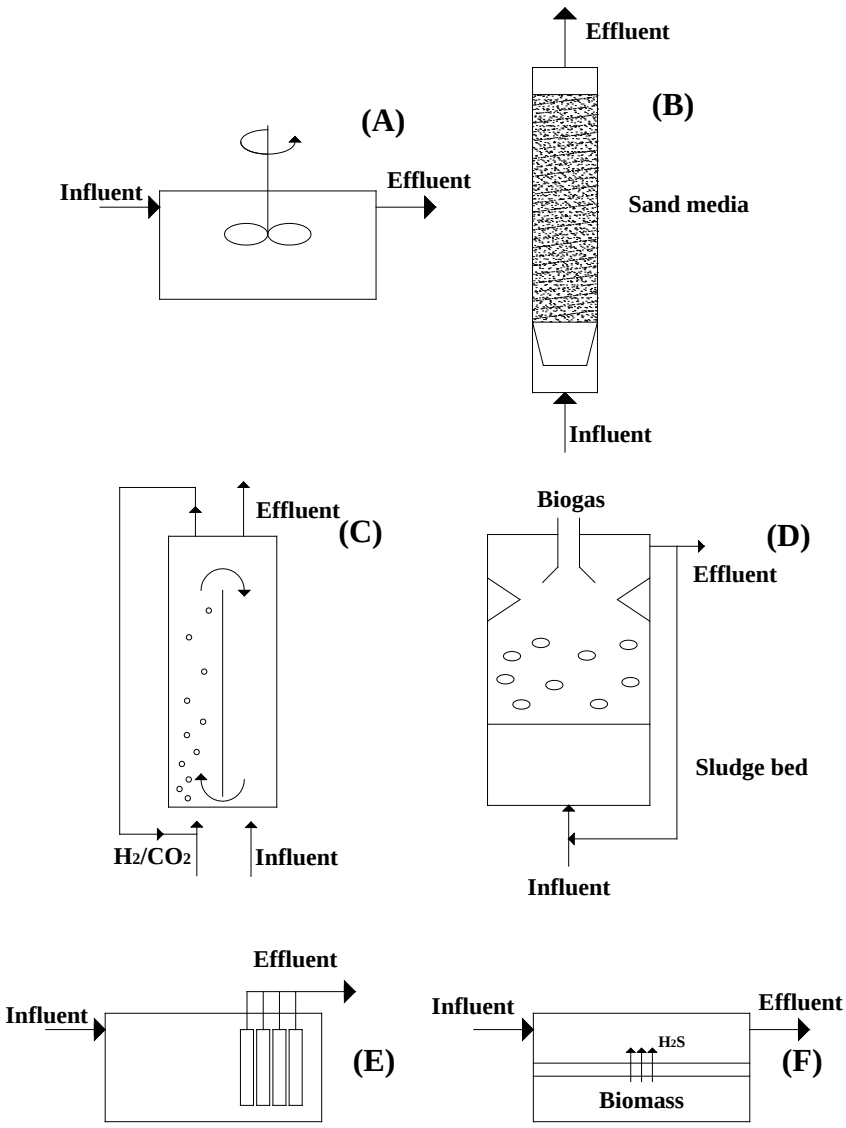

Figure 4 - Schematic representation of a CSTR (A), a PBR (B), a GLR (C), a UASB reactor (D), an immersed membrane bioreactor (IMBR) (E) and an extractive membrane bioreactor (EMBR) (F)

Continuously stirred tank reactors (CSTRs) have been shown to be very consistent and reliable but the risk of washout of the active biomass is frequent

(Lens et al., 2003). To produce a minimal quantity of sludge and to reach a good sludge stability, CSTRs should be operated at high sludge retention time (SRT), but this leads to a significant increase in reactor volume and thus capital costs.

On equal reactor volumes, packed-bed reactors (PBRs) guarantee a higher SRT than CSTRs (Barnes et al., 1991). The most important disadvantages of PBRs are due to the clogging of the bed by precipitates and filtered particles and the utilization of high pressures for pumping the water through the reactor (Anderson et al., 1990). This reactor configuration can be operated both in down-flow mode, taking advantage of the gravity and reducing the operating costs (Zaluski et al., 2003), or in up-flow mode (Jong and Parry, 2003).

Gas-lift reactors offer three important benefits: *a)* high mass transfer of the substrates into the bacterial agglomerates; *b)* a very good mixing of the reactor liquid reducing the negative effects of possible toxic compounds and *c)* high rate biological kinetics because of the use of H_2 as electron donor (van Houten et al., 1994; van Houten et al., 1997; Dijkman and Buisman, 1999; Esposito et al., 2003). Drawbacks of gas-lift reactors are the rather high operating costs linked to the pumping of the gaseous substrates (Lens et al., 2002).

The most important peculiarity of up-flow anaerobic sludge blanket (UASB) reactors is the very good settling capability of the biomass: microorganisms form a granular sludge-bed the influent passes through (Lettinga et al., 1980; Omil et al., 1996). Compared to PBRs, no carrier material is necessary and there are no problems of clogging: therefore start-up and operating costs are appreciably lower. The main disadvantages of UASB reactors are the washing out of the biomass during process failures and the high susceptibility to changes in the influent quality if compared to other bioreactor typologies such as PBRs (Jhung and Choi, 1995).

The use of membrane bioreactors has gained a lot of interest in recent years (Mack et al., 2004). Membranes are often immersed inside the bioreactor (IMBR), which overcomes the need for a sedimentation basin tank to recirculate the biomass. This bioreactor configuration enhances biomass retention compared to suspended culture bioreactors and promotes the development of a performing biomass in terms of substrate degradation as well (Mack et al., 2004). For acid mine drainage treatment, an extractive membrane biological reactor (EMBR) is preferred in order to prevent the direct contact between SRB and toxic metals (Chuichulcherm et al., 2001) Metal-containing wastewater passes through one surface of the membrane, while bacteria are kept in suspension on the other side (Manconi and Lens, 2009). The membrane prevents metals to get in contact with biomass but allows biogenic H_2S to pass in the opposite direction to induce metal precipitation (Mack et al., 2004). Disadvantages of membrane bioreactors are related to *i)* the high costs to overcome the trans-membrane pressure and *ii)* the fouling due to the deposition of microbial aggregates and metal precipitates on the surface of the membrane (Fedorovich et al., 2000; Chuichulcherm et al., 2001). A periodic backwash is needed to clean the membranes (Tabak and Govind, 2003b; Vallero et al., 2005).

2.2.4.2 Interaction of SRB with other microbial species

SRB compete with methanogenic archaea (MA) and homoacetogenic bacteria (HB) for organic (Weijma and Stams, 2001; Celis-Garcia et al., 2007) and inorganic (Esposito et al., 2009; Frunzo et al., 2012) electron donors in anaerobic environments. When hydrogen is supplied to a GLR, although H_2 is an attractive electron donor for all the three microbial groups, SRB have been shown to outcompete MA and HB (Weijma et al., 2002). SRB can outcompete

methanogens also for acetate utilization, when sulfate is not the limiting factor (Bhattacharya et al., 1996). However, SRB do not generally prefer acetate as electron donor (Koschorreck et al., 2004). Therefore, the competition for acetate also depends on the availability of other carbon sources available, even if sulfate is added in excess (Stams et al., 2005).

The fate of methanol in anaerobic reactors is determined by the outcome of competition among SRB, MA and HB as well. SRB can outcompete the other species when the temperature is very high (T = 65°C). On the contrary, under mesophilic conditions, 90% of methanol has been observed to be converted to methane (Weijma et al., 2000; 2003).

Propionate and butyrate oxidation can follow the sulfate reduction way, when sulfate conditions are not limiting. At high sulfate concentrations, propionate is effectively degraded by SRB, whereas butyrate oxidizers well compete with sulfate reducers even with an excess of sulfate (Visser et al., 1993).

2.2.4.3 Metal sulfide precipitation

When sulfidogenic treatment is applied to AMD, metal can be removed from solution as metal sulfide particles (Alvarez et al., 2007). The stoichiometry between sulfide and metals should be carefully controlled, because the unreacted sulfide remains in solution and needs to be removed (Veeken et al., 2003a). Furthermore, sulfide to metal ratio affects the size and the location of metal sulfide precipitates (Villa-Gòmez et al., 2011). Figure 5 shows two particulars of Sotkamo mine in Finland: *i)* the soil heaps where metal bioleaching occurs and *ii)* the reactors for metal sulfide precipitation.

Figure 5 - Particulars of Sotkamo mine: on the left, the heaps of soil interested by bioleaching of metals; on the right, the metal sulfide precipitation reactors for metal recovery from leaching solution

The metal sulfides initially form very small fines and then small crystals in solution (Mersmann, 1999). The more the particles tend to agglomerate, the more effective the sedimentation process is (Veeken et al., 2003a). The particles size distribution is dependent by the rate of crystal growth and nucleation which are controlled by supersaturation. The crystal size decreases as the supersaturation of the solution increases (Mersmann, 1999). When the precipitating component is added at a fast rate or in excess to the stoichiometric ratio, very small particles are formed and they tend not to settle coming out of the reactor with the effluent (Villa-Gòmez et al., 2011).

Biological sulfate reduction and metal precipitation using biogenic H_2S can be applied in single or separated unit processes (Figure 6). The single-stage treatment process (Figure 6A) is a low-cost solution for AMD treatment, but it could not be feasible when acidic wastewaters with high heavy metal concentrations are fed to the bioreactor (Tabak et al., 2003a). One of the solution could be to use alkaline compounds to generate additional alkalinity in order to promote the bacterial activity (Figure 6B) (Hammack and Edenborn,

1992). Another approach could be to recycle part of the treated water (Figure 6C) to dilute the influent by using, for instance, particular reactor configurations such as fluidized-bed reactors (Maree and Strydom, 1987; Ma and Hua, 1997; Villa-Gòmez et al., 2011). However, both the solutions require additional investment and operational costs (Hao, 2000). Furthermore, in a single-stage process, sulfide concentration is not easy to control. It would be convenient to maintain sulfide concentration rather high to buffer the system against possible metal shock loads. On the other hand, too high dissolved sulfide levels may be toxic for SRB (O'Flaherty et al., 1998).

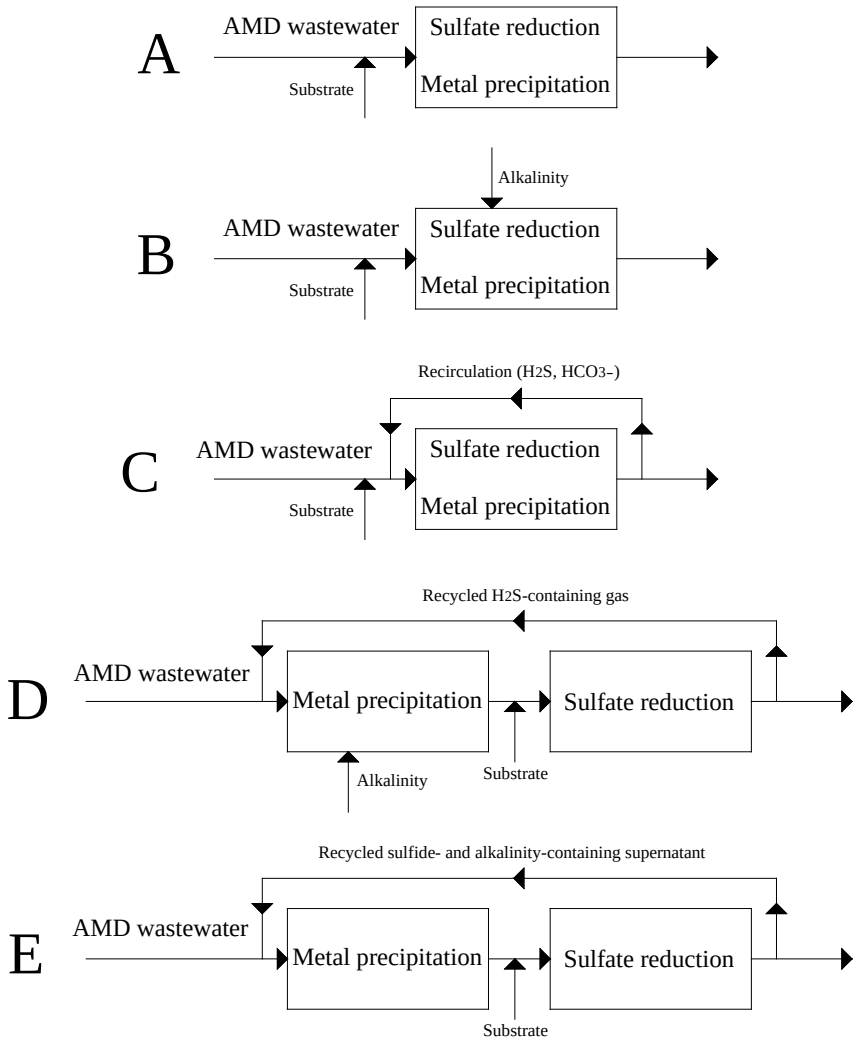

Figure 6 - Possible configurations of processes utilizing sulfate-reducing bioreactors for metal precipitation

To avoid the direct contact between SRB and heavy metals, metals can be precipitated and settled in a special basin prior to the biological phase by recycling the biogenic sulfide (Govind et al., 2001). An “ad-hoc” phase for metal precipitation is required, besides the installation of more pumps and lines.

Therefore, this treatment solution is not as cost effective as the single-stage one (Govind et al., 1997). Sulfide can be recycled back to the metal precipitation reactor as sulfide solution or H_2S-containing gas (Bhagat et al., 2004). Recycling sulfide in a gas stream (Figure 6D) promote a better selective precipitation of metals, since no alkalinity is introduced to the precipitation step and the precipitates only consist of metal sulfides. However, metal sulfide precipitation produces acidity, further decreasing the pH of the influent (reaction 12). Therefore, an external source of alkalinity has often to be used when sulfide is provided to the metal precipitation step as H_2S gas (Hammack et al., 1993). Using sulfide-containing liquid supernatant (Figure 6E), higher metal removal efficiencies can be achieved than sparging the solution with H_2S. Furthermore, alkalinity has not to be added externally because bicarbonate are recycled back with the supernatant. On the contrary, metals do not only precipitate as sulfides but also as unidentified complexes including nitrates, chlorides and carbonates (Bhagat et al., 2004).

2.2.4.4 Metal recovery

Metals are non-renewable resources and thus metal recovery and reuse from wastewaters is beneficial for economic and environmental reasons (Badmus et al., 2007; Fu and Wang, 2011). Metal recovery from multiple metal-AMD wastewaters can be performed using one of the treatment solutions shown in Figure 6. Sulfide concentration and pH are the most important parameters to control for achieving selective metal precipitation with the highest purity degrees (Tabak et al., 2003a). For a better control of sulfide concentration and pH in the precipitation phase, it is advisable to use a multiple-stage process separating the biological process from the metal precipitation step. Cu, Zn, Al and Fe have been shown to be selectively precipitated in a four-stage process by

using different sulfide levels (Tabak et al., 2003a). Similarly, a 100% sequential precipitation of Cu and Zn has been obtained at pH 2 and 3 in a CSTR at very low sulfide concentrations (Sampaio et al., 2009).

Selective metal recovery can be also performed successfully in a single-stage process, when the precipitation of two or more different metals can be attained by only controlling the pH. NiS has been observed to be selectively precipitated from a nickel-iron solution at pH of 5, whereas, increasing the solution pH to 5.5, iron sulfide precipitates as well generating a heterogeneous Ni-Fe sludge (Bijmans et al., 2009).

2.3 BIOLOGICAL DENITRIFICATION

Nitrate (NO_3^-) is the most common groundwater contaminant (Korom, 1992). Nitrate pollution widely increased in the seventies, because of growing anthropogenic sources especially in agriculture, such as the large application of N fertilizers, the mismanagement of irrigated crops and the disposal of livestock waste (Freeze and Cherry, 1979; Hallberg, 1989). The uncontrolled loss of landfill leachates often results in an important nitrate contamination of groundwater and soil as well. Surface waters can be also characterized by high nitrate pollution, because of the discharge of untreated industrial effluents from mining and extractive industry (Zaitsev et al., 2008). Nitrate in blasting agents can dissolve in water from undetonated explosives or can be produced as a result of the large use of N-containing leaching agents such as cyanide used for metal extraction (Forsberg and Åkerlund, 1999; Zagury et al., 2004).

As well as for metal and sulfate removal, several technologies exist for nitrate reduction in water. However, biological denitrification seems to be the most effective one for nitrate-contaminated wastewater, in terms of removal efficiencies and operating costs (Kapoor and Viraraghavan, 1997).

2.3.1 Principles

Biological denitrification is the primary process for removal of nitrate from water and soil by reduction to nitrogen gas, through various gaseous inorganic forms (Figure 7) (Knowles, 1982). Denitrification consists of four steps (Maier et al., 2000):

- reduction of nitrate to nitrite (NO_2^-), catalyzed by the enzyme nitrate reductase, strongly inhibited by free oxygen.

- conversion of nitrite to nitric oxide (NO), catalyzed by the nitrite reductase enzyme. Nitrite reductase is unique to denitrifying organisms and its synthesis is inhibited by oxygen and induced by nitrate.

- conversion of nitric oxide to nitrous oxide (N_2O). This step involves a third enzyme called nitric oxide reductase. The synthesis of this enzyme is inhibited by oxygen and induced by various nitrogen oxide forms.

- reduction of nitrous oxide to nitrogen gas (N_2), catalyzed by the nitrous oxide reductase enzyme. The activity of this enzyme is inhibited by low pH and even more sensitive to oxygen than the other three enzymes in the denitrification pathway.

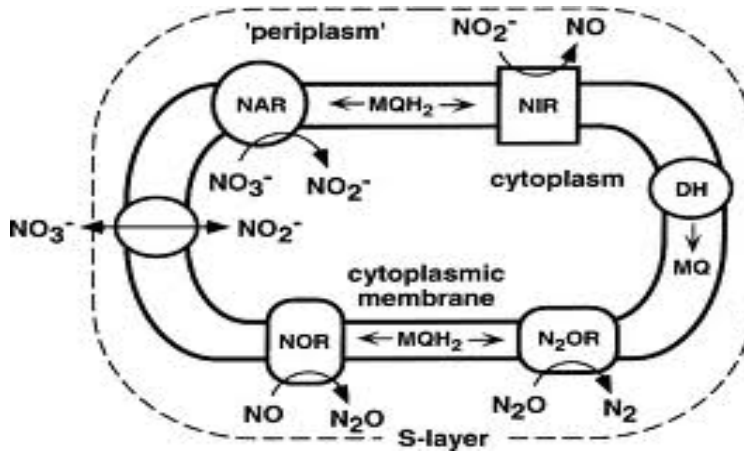

Figure 7 - Denitrification pathway

Generally, low nitrate levels promote the accumulation of nitrous oxide as end product. The greenhouse effect of N_2O is approximately 300 times more potent than carbon dioxide and N_2O can cause depletion of the ozone layer contributing to global warming (Ravishankara et al., 2009). On the contrary, higher nitrate

concentrations lead to the complete extent of denitrification, with nitrogen gas as more desirable final product.

NO_3^-, NO_2^-, NO and N_2O are all used by denitrifying bacteria as electron acceptors. The organisms capable of conducting denitrification are found widely in the environment and display a variety of different characteristics in terms of metabolism and activities (Tiedje, 1982a; Maier et al., 2000). The majority of denitrifiers are heterotrophic but several autotrophic denitrifying bacteria have been identified, using H_2 (Lee and Rittmann, 2003) or iron and sulfur (Zhang et al., 2012) as electron donors. Table 10 shows different species of denitrifiers:

Table 10 - Genera of denitrifying bacteria (Myrold, 1998)

Genus	**Characteristics**
Heterotrophs	
Alcaligenes	Common soil bacterium
Agrobacterium	Plant pathogens
Aquaspirillum	Magnetoactic, oligotrophic
Azospirillum	Associative N2 fixer, fermentative
Bacillus	Spore former, fermentative, some species thermophilic
Flavobacterium	Common soil bacterium
Halobacterium	Halophilic (high salinity tolerant)
Hyphomicrobium	Grows on one C-substrates, oligotrophic
Kingella	Animal pathogen
Neisseria	Animal pathogen
Propionibacterium	Fermentative
Pseudomonas	Isolated from soil, very diverse genera
Autotrophs	
Alcaligenes	Uses H_2, common soil isolate
Hydrogenophaga sp.	H_2 utilizer

Pseudomonas	Uses H_2, common soil isolate
Thiobacillus	Uses H_2 and reduced sulfur compounds

Besides denitrification, nitrate can also be reduced through a different process known as dissimilatory nitrate reduction to ammonium (DNRA). It uses nitrate as terminal acceptor and leads to the production of ammonium as end product. DNRA bacteria are predominantly heterotrophic and DNRA is strongly favored over denitrification in organic-rich environments (Tiedje et al., 1982b) On the contrary, denitrification provides more energy per mole of nitrate reduced than DNRA. Thus, in a carbon-limited environment, denitrification will be the preferred pathway for nitrate reduction (Tiedje et al., 1982b).

Finally, a different fate for nitrate is the uptake into living biomass by plants and microorganisms. The uptake of nitrate is followed by its reduction to ammonium, which is then incorporated into biomass. This process is called assimilatory nitrate reduction or nitrate immobilization.

2.3.2 Electron donors

Many organic or inorganic compounds have been tested in literature as electron donor/carbon source for denitrification. Most of these substances are liquid (i.e. ethanol, methanol and acetate) but solid (i.e. biodegradable polymers, straw, reed, birch wood) and gaseous (i.e. methane and hydrogen) have been used as well (Mateju et al., 1992). There are several factors that have to be considered in the choice of a carbon source such as cost, denitrification rates, kinetics, degree of utilization, sludge production, handling and storage safety/stability (Æsøy et al., 1998).

Solid carbon-rich substrates are used either as carbon source and support for the biofilm growth. In municipal wastewater treatment plants, the use of solid electron donors can limit the release of organics to the following disinfection process, avoiding the formation of disinfection by-products such as trihalomethanes. Moreover, most of these materials are cheap waste products from agriculture and forestry (Ovez et al., 2006) or sewage sludge and solid organic wastes from households and industry (Æsøy et al., 1998). Recently, the development of water-insoluble biodegradable polymers led to the idea to use such materials in denitrification reactors (Mergaert et al., 2001). Poly 3-hydroxybutyrate (PHB) and poly caprolactone (PCL) have been tested for denitrification and pesticide elimination in drinking water (Mergaert et al., 2001; Boyer et al., 2003).

Liquid and gaseous substrates have been more used than solid compounds for denitrification applications. The most important advantage of using liquid or gaseous compounds is due to their easier uptake into microbial cells and faster degradation, resulting in higher denitrification rates (Bandpi and Elliott, 1998). However, the installation of complex dosing systems is often needed for supplying the soluble organic compounds to the reactors.

Methanol, ethanol and acetate are the most used liquid substrates for denitrification (dos Santos et al., 2004; Tartakovsky et al., 2007; Calderer et al., 2010). Methanol has been commonly used in full-scale wastewater treatment plants as external carbon source especially due to its low cost (Louzeiro et al., 2002). However, in some countries ethanol is a valuable alternative since it is largely produced from sugar cane and costs less than other carbon sources (dos Santos et al., 2004). In the last years, the role of methane for denitrification has been investigated, since methane is highly produced in anaerobic reactors (Thalasso et al., 1997).

Generally, ethanol has found to be the most appropriate carbon source for denitrification purposes. Its utilization results in higher denitrification rates, faster microbial acclimation and lower C/N ratios needed if compared to methanol (Christenson et al., 1994; dos Santos et al., 2004). Moreover, ethanol has been shown to be very suitable to promote denitrification even at 5°C (Martin et al., 2009).

Denitrification can be efficiently maintained by using acetate as carbon source with a C/N ratio between 6 and 9. However, denitrification performance with acetate is lower than with ethanol as it leads to slower acclimation of microbes and higher nitrite accumulation (Martin et al., 2009).ù

The first studies reporting methane utilization for denitrification date back to 1970s. Davies et al. (1973) found that denitrifying bacterial species only enriching on methane do not exist. Methane is first converted to methanol aerobically and methanol is used as electron donor by denitrifying bacteria for nitrate removal. Therefore, a small amount of oxygen is needed in solution to promote methane conversion to methanol and then denitrification (Thalasso et al., 1997). However, nitrate removal efficiencies and denitrification rates are lower than with different carbon sources.

Finally, denitrification can be carried out using autotrophic biomass as well. H_2 is an excellent autotrophic choice because of its clean nature, low biomass yield and low sludge treatment, relatively low cost as well as it does not persist in the treated water (Lee and Rittmann, 2002). Low H_2 solubility in water is the most important shortcoming that limits H_2 utilization for denitrification purposes.

2.3.3 Environmental factors

2.3.3.1 Dissolved oxygen

Oxygen is strongly inhibiting for denitrification. Oh and Silverstein (1999) found that an oxygen concentration of 0.09 mg/L already inhibited the activity of enzymes, resulting in a 35% of denitrification rates. Nitrous oxide reductase is the most sensitive enzyme. In fact, oxygen presence in water especially limits nitrous oxide conversion to nitrogen gas (Baumann et al., 1996).

2.3.3.2 pH

The optimum pH range for complete reduction of nitrate to nitrogen gas is considered to be between 6 and 8. Below this optimal pH, nitrate reduction occurs but nitrous oxide may be the final product of denitrification (Knowles, 1982). The low pH inhibition is due to the accumulation of nitrite in solution (Glass and Silverstein, 1998). At low pH, nitrite exist in the protonated form as nitrous acid (pK_a=3.7) that has been shown to inhibit denitrification at concentration as low as 0,04 mg/L (Abeling and Seyfried, 1992). However, bacteria adapted at very low-pH conditions (pH=2.6) and capable of reducing nitrate have been found (Baeseman et al., 2005).

2.3.3.3 Temperature

In most scientific studies, denitrification has been observed to be strongly affected by temperature. Denitrification develops better at temperatures higher than 16°C and the optimal temperature has been observed around 30°C (Amatya et al.,, 2009). However, many prychrotolerant organisms are capable of reducing

nitrate at low temperatures, even though they show an optimal temperature of 20°C (Madigan et al., 1997). Welander and Mattiasson (2003) observed that denitrification had only a rather weak dependence on the temperature in a nitrate-reducing MBBR operated between 3 and 15°C.

2.4 FLUIDIZED-BED REACTORS

Fluidized-bed reactors (FBR) are an important alternative biofilm process for biological wastewater treatment (Nikolov and Karamanev, 1991; Papirio et al., 2012b). In FBRs, a recirculation flow fluidizes small carrier particles and induces extensive cell immobilization, thereby achieving a high reactor biomass hold-up and a long cell residence time (Shieh and Hsu, 1996). Besides heavy metal removal, sulfate reduction and denitrification, FBRs have been applied abundantly for nitrite removal (Boehler and Haldenwag, 1991), anaerobic digestion (Heijnen et al., 1989; Anderson et al., 1990; Garcia-Calderon et al., 1998a; Buffiere et al., 2000; Arnaiz et al., 2003; Sowmeyan and Swaminathan, 2008a; 2008b) and the removal of chlorinated phenols (Puhakka and Järvinen, 1992; Wilson et al.,1997), chlorinated aliphatic compounds (Niedzielski et al., 1989) and aromatic hydrocarbons (Shimodaira and Yushina, 1983; Voice et al., 1992).

2.4.1 Fluidization typologies

Fluidization can be conducted in up-flow and down-flow modalities (Figure 8). In the traditional up-flow fluidization, the solid particles have a higher density than the liquid and are fluidized by the liquid stream in the opposite direction of gravity. The up-flow fluidized-bed bioreactor (UFBR or simply FBR) design has a series of advantages compared to other anaerobic reactor concepts (Iza, 1991):

- an efficient biomass retention that allows high mass transfer and reaction rates (Speece, 1983; Shieh and Keenan, 1986; Marin et al., 1999);
- higher organic loading rates (OLRs) and lower hydraulic reaction times (HRTs) permit lower reactor sizes (Garcia-Calderon et al., 1998a);

- large surface area for biofilm formation due to the fluidization of the bed (Anderson et al., 1990; Diez-Blanco et al., 1995);
- good mixing and contact between substrate and biomass (Nicolella et al., 1997);
- great resistance to inhibitors due to the recycle flow that dilutes high influent concentrations and acidity (Marin et al., 1999; Kaksonen et al., 2003a; 2003b; 2004b; Sahinkaya et al., 2007a, 2007b);
- high efficiencies in terms of sulfate reduction (Celis-Garcia et al., 2007) and metal precipitation (Kaksonen et al., 2003a; Sahinkaya 2007b).

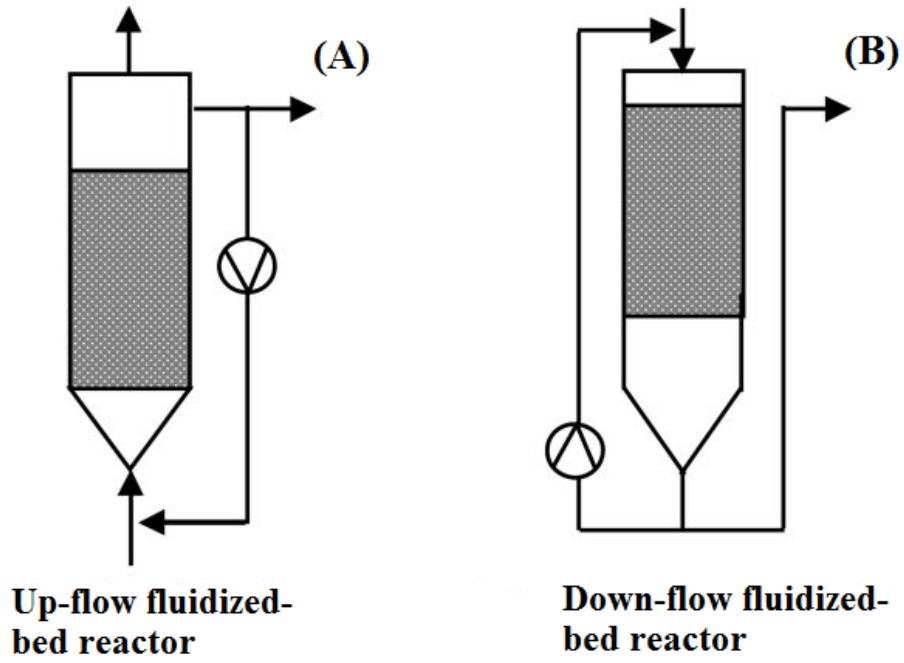

Figure 8 - Schematic representation of an up-flow FBR (A) and a down-flow FBR (B)

The inverse (or down-flow) fluidized-bed reactor (IFBR or DFFBR) utilizes small particles with a lower specific density than the specific density of the

water, thus particles float and are expanded downward by the liquid flow (Sowmeyan and Swaminathan, 2008b). In the case of gas production, it also contributes to bed expansion and this phenomenon is called pseudo-fluidization (Arnaiz et al., 2003). However, other authors have found that gas formation has just a little effect on the hydrodynamic behavior, and therefore it is possible to describe the bed in an IFBR like a two-phase solid-liquid system (Diez-Blanco et al., 1995). Down-flow fluidization has further advantages compared to traditional fluidization such as:

- it allows the recovery of solid products, such as metal sulfides, at the bottom of the reactor. In this way, the biofilm developing on the top of the reactor remains separated from the metal precipitates (Celis-Garcia et al., 2007; Kaksonen and Puhakka, 2007; Villa-Gòmez et al., 2011);
- it is not prone to clogging (Garcia-Calderon et al., 1998b);
- it has a lower energy requirement (Castilla et al., 2000).

2.4.2 Carrier materials adopted in fluidized-bed applications

Table 11 shows the characteristics and the fluidization velocities of different materials used as support for the growth of the microbial species in several fluidized-bed applications. Many materials, with density higher than water, such as porous glass beads, granular activated carbon (GAC), silicate mineral sand and celite particles have been used and tested as packing materials in classical FBRs, showing all good biomass attachment capabilities with different reactor operating conditions.

Table 11 - Fluidization velocities of different carriers used in up-flow and down-flow fluidized-bed reactors treating different types of wastewater (divided by fluidization typology)

Carrier	v (m/h)	Application	Author
Up-flow FBR			
Mineral Manville Celite R-633 particles	9.2-12.8	Aerobic treatment of polychlorinated phenols	Puhakka and Järvinen (1992)
0.75 mm granular activated and non-activated carbon	17.4-36.6	Treatment of volatile aromatic hydrocarbons	Voice et al. (1992)
0.86 mm sand particles	16.8-56	Denitrification	Green et al. (1994)
Polymeric granules cover with iron dust	186	Sulfate reduction	Somlev and Tishkov (1992)
0.1-0.2 cm porous glass beads	145.8-151.2	Sulfate reduction	Nagpal et al. (2000a)
0.5-1 mm silicate mineral Filtralite sand	29	AMD treatment	Kaksonen et al. (2004b)
Down-flow FBR			
4.3 mm polyethylene cylinders	18-28	Hydrodynamic study	Hihn (1992)
3.2 mm synthetic foam cylinders	20-30	Hydrodynamic study	Hihn (1992)
3.6 mm polyethylene spheres	8.6-13.2	Hydrodynamic study	Garcia-Calderon et al. (1998b)
0.92 mm cork particles	6.2	Hydrodynamic study	Garcia-Calderon et al. (1998b)
3.6 mm foamed polypropylene spheres	39	Aerobic treatment of oil refinery wastewater	Shimodaira and Yushina (1983)
2-6 cm foamed polystyrene particles	45-60	Nitrite removal	Boehler and Haldenwag (1991)
0.968 mm perlite particles	2.3	Anaerobic digestion of wine distillery wastewater	Garcia-Calderon et al. (1998a; 1998b)
0,175 mm spherical granular silica particles	5.4	Anaerobic digestion of dairy wastewater	Arnaiz et al. (2003)
0.4 mm low density	10.9	AMD treatment	Celis-Garcia et al.

polyethylene pellets			(2007)
0.5 mm low density polyethylene fine particles	18.6	AMD treatment	Celis-Garcia et al. (2008); Gallegos-Garcia et al. (2008)

Spherical glass beads have been shown to be very suitable as carrier material due to their roughness and porosity characteristics of the surface (Nagpal et al., 2000a). Numerous craters (10-30 μm wide and 5 μm deep) on the surface of this material make the glass beads suitable for biomass attachment and growth. Using scanning electron microscopy, uniform biofilms develop over the entire surface area and not only in the biggest crevices of GAC. This results from the ability of the activated carbon to concentrate nutrients necessary for microbial growth and to provide a well-protected environment from fluid shear forces (Voice et al., 1992)

Sand particles have been shown to guarantee a very good biomass attachment as well. Kaksonen et al. (2003a) could test their sulfate-reducing and metal-precipitating FBRs by increasing the recirculation flow up to 30% using a silicate filtralite support. Similarly, Puhakka and Järvinen (1992) observed that the microbial cells entrapped within the pores of a celite carrier were well protected from the shear forces and carrier collisions. The celite carrier also showed to have a high persistence to the mechanical friction caused by the up-flow velocity. In a denitrifying fluidized bed treatment, Green et al. (1994) observed an unexpected biofilm thickness growth (up to 200 μm). The excessive growth of microorganisms increases the biofilm thickness which limits diffusion of the substrate to the deeper biofilm layers. Starvation of microorganisms at the base of the biofilm causes the detachment of pieces of biofilm and leads to ineffective bioreactor operation. In anaerobic conditions, the maximum biofilm thickness at which no diffusional limitation occurs has been observed to be about 100 μm in a liquid-solid IFBR (Karamanev and Nikolov, 1996).

In down-flow fluidization, floatable materials such as polyethylene, polypropylene, polystyrene, perlite and cork are used (Garcia-Calderon et al., 1998b; Castilla et al., 2000). In IFBRs biomass accumulation makes particles heavier, increasing particle density and bed expansion. If there is an excess of biomass accumulation, the density of the particles can attain 1000 kg/m^3 and particles may be washed out from the reactor (Buffiere et al., 2000). Bacteria which carry out the anaerobic digestion of wastewater, stick to the fluidized particles, modifying their density, size, shape and therefore the hydrodynamic behavior. Thus, it is important to fit the recirculation ratio to the amount of attached microorganisms in order to maintain a fixed expansion of the bed (Garcia-Calderon et al., 1998a). Celis-Garcia et al. (2008) showed that at lower superficial velocities (10 m/h), a high amount of biomass (1.29 gVSS/l) attached to the upper part of the plastic support where the fluidization was lower, while no biofilm grew in the lower part of the bed where the fluidization was higher. Increasing the superficial liquid velocity up to 15.2 m/h, a uniform expansion of the carrier material was achieved but leading to detachment of part of the biomass (0.80 gVSS/l).

Biomass accumulation is not the only parameter affecting bed expansion because other substances can precipitate onto the surface of the carrier material. Villa-Gòmez et al. (2011) observed that metal precipitates can be located in the biofilm especially when the sulfide concentration is low in the reactor mixed liquor. In these conditions, the supersaturation and the precipitation of the metal sulfide fines occur in the biofilm because sulfide is present in higher concentrations around its surface.

Garcia-Calderon et al. (1998b) tested different carrier materials and calculated the minimum fluidization velocities: perlite particles (Φ=0.968mm, v=2.3m/h), polyethylene spheres (Φ=3.6mm, v=13.2m/h), polypropylene spheres

(Φ=3.6mm, v=8.6m/h) and ground cork particles (Φ=0.92mm, v=6.24m/h). Perlite was chosen among the four materials due to the lowest minimum fluidization velocity and due to the presence of sharp angles and crevices on its surface where microorganisms tend to grow up better (Garcia-Calderon, 1998a).

Celis-Garcia et al. (2007) used low density polyethylene pellets with 0.4mm mean diameter as biomass carrier material for an IFBR, without any kind of treatment of the polyethylene surface. Choi and Shin (1999) mentioned that polyethylene has hydrophobic surface properties, which are not good for the immobilization of microbes. Therefore, they modified the surface properties of the polymer substrate from hydrophobic to hydrophilic by treating the polyethylene surface with chlorosulfonic acid. To increase the polyethylene surface area, Villa-Gòmez et al. (2011) crushed 3 mm diameter-beads with sand in a blender promoting a better biomass attachment.

2.4.3 Fluidized-bed applications for sulfate reduction and metal removal

Many authors have studied the feasibility of removing sulfate and heavy metals from AMD using FBRs and IFBRs. The influence of operational conditions on fluidized-bed treatment of AMD is described as follows. In Table 12 the operational conditions adopted in many sulfate- and metal-treating FBR studies are summarized.

2.4.3.1 Fluidization degree

The fluidization degree of the carrier affects the start-up and maintenance of the FBR-process in a different way. In terms of sulfate reduction, sulfide production and effluent alkalinity, the start-up phase of a FBR, using silicate mineral sand

as carrier material, with a 10% fluidization degree has been shown to be superior to FBRs operated at 20% and 30% fluidization degrees (Kaksonen et al., 2003a). At low recycling rates, smaller attrition supports faster biofilm growth on the carrier material promoting a faster increment in reactor performance. On the contrary, high recycling rates allow the treatment of low pH and high metal concentration wastewaters because of the better dilution of acidity and metal loads. Kaksonen et al. (2003a) observed that the FBRs performed better at the highest loading rates (sulfate loading rate = 3400 ± 200 mg/L·day, organic loading rate = 880 ± 20 mg/L·day) with 20-30% fluidization degrees reaching about 80% of sulfate reduction efficiency. Moreover, the low feed pH (2.5 ± 0.1) was completely neutralized and 230 mg/L of Zn was totally precipitated.

2.4.3.2 pH

Strong acidic wastewaters (pH=2.5-3) like AMD can be completely neutralized to 7.5-8.5 by the alkalinity produced by SRB during lactate or ethanol oxidation in a FBR (Kaksonen et al., 2003a). This is possible due to the alkalinity produced by the SRB and the recycle flow that dilutes influent concentrations and acidity so that SRB are active in a pH range close to that of the treated effluent.

2.4.3.3 Temperature

Low temperature has been shown to strongly affect sulfate-reducing activity in FBRs. Biogenic alkalinity is hardly produced at psychrophilic conditions (T=8°C) because of the incomplete oxidation of the electron donor (lactate or

ethanol) to acetate, thus resulting in the need for an external $NaHCO_3$ addition to the feed solution in order to guarantee pH conditions favorable for the bacteria (Sahinkaya et al., 2007a). The same authors observed that the performance of the biological process did not reach high COD removal and sulfate reduction efficiencies, despite the inoculation of several low-temperature cultures of SRB enriched on a mix of ethanol and lactate (Karnachuk et al., 2005).

At thermophilic conditions (65°C), sulfate-reducing activity has been shown to be faster (Sahinkaya et al., 2007a). They observed a 99.9, 46 and 29% ethanol, sulfate and acetate removal efficiency, respectively, just after 6 days of reactor operation. The average sulfate reduction and acetate oxidation rates were three and four times higher, respectively, at 65 than at 8°C. The biological process was carried out by particular species of thermophilic SRB (Kaksonen et al., 2006) that showed higher degradation yields in long-term operation but, however, did not respond immediately to sudden changes in influent pH and loading rates (Sahinkaya et al., 2007b).

Both at psychrophilic and thermophilic conditions, enough biogenic sulfide can be produced in FBRs to achieve a complete metal precipitation. In the study of Sahinkaya et al. (2007a), iron was completely removed as iron sulfide at loading rates of 90 and 60 mg/L·day at 65°C and at 8°C, respectively.

Table 12- Operational conditions in FBRs and IFBRs aimed at the treatment of sulfate- and metal-containing AMD wastewaters

Fluidization degree (%)	Temperature (°C)	HRT (h)	Organic carbon source	Organic loading rate (g/L · day)	Sulfate loading rate (g/L · day)	COD/SO_4^{2-} (g/g)	Metal loading rate (mg/L · day)	Reference
-	-	5-55	Lactate/ethanol	1.7-9.6	0.87-12	0.8-1	-	Nagpal et al (2000a)
10-20-30	35 controlled	16	Lactate (DOC)	0.65-0.88	2.45-3.4	0.2	350 Zn 86 Fe	Kaksonen et al. (2003a)
20	35 controlled	16	Lactate (DOC) Ethanol (DOC)	0-0.25 0-0.38	1.6-3.7	0.11 0.11	500 Zn 85 Fe	Kaksonen et al. (2003b)
20	35 controlled	6.1-20.7	Ethanol (DOC)	0.8-2.7	2.32-7.87	0.3	200-650 Zn 115-400 Fe	Kaksonen et al. (2004b)
20	8 and 65 controlled	24	Ethanol (DOC) Acetate	0.32-0.48 0.5-0.7	1-1.5	0.32-0.38	45-100 Fe	Sahinkaya et al. (2007a)
20	8 and 65 controlled	24	Acetate	0.5-0.7	1	0.5-0.7	-	Sahinkaya et al. (2007b)
30-40	30 controlled	16.8-24	Mixture of VFA (COD)	2.5-5.2	1.5-7.3	0.67-1.67	-	Celis-Garcia et al. (2007)
50	18-26 (room temperature)	24-48	Ethanol/lactate (COD)	2.5	1.5-3	0.8-1.67	320 Fe 220 Zn, 20 Cd	Gallegos-Garcia et al. (2008)
25	25±3 (room temperature)	48	Ethanol/lactate (COD)	0.5-1	0.83-1.66	0.6	-	Celis-Garcia et al. (2008)
25	Room temperature	9-24	Lactate (COD)	5-13.33 1-2.67	1-2.67	5 1	5-27 Zn, Cu, Pb, Cd	Villa-Gòmez et al. (2011)

2.4.3.4 HRT

Several studies assessed the effect of the HRT on a FBR treating a heavy metal and sulfate containing wastewater. In almost all the studies, a higher HRT is applied at the beginning of the experiments to enhance the contact time between the microorganisms and the support (Celis-Garcia et al., 2008). This results in a significant biomass immobilization on the carrier material. Then, in order to test the robustness of the reactors, the HRT is quickly or gradually decreased. If a significant biofilm development is attained on the carrier support, pseudo-steady states are reached in a short period of time after operational HRT changes (Nagpal et al., 2000a; Celis-Garcia et al., 2007).

FBRs operated at an HRT as low as 6.5 h have been demonstrated to successfully remove zinc and iron from an acidic simulated mine water if the HRT is gradually decreased (Kaksonen et al., 2004b). However, a sudden decrease of the HRT from 9.7 h to 7.3 h resulted in a decrease of the reactor performance since higher metal concentrations were detected in the effluent solution. Effluent pH and sulfide concentration decreased from 8 to 5 and from 200 to 50 mg/L, respectively, whereas effluent iron concentration increased up to 10 mg/L (Kaksonen et al., 2004b). In contrast, when the feed pH is closer to neutrality, a sudden change of HRT affects less the AMD fluidized-bed treatment (Villa-Gòmez et al., 2011). At low HRTs, the incomplete oxidation of the electron donor is easier resulting in high acetate effluent concentrations (Kaksonen et al., 2004b). Under psychrophilic and thermophilic conditions, SRB have been shown to be more sensitive to HRT changes. Too quick HRT decreases could lead to the process failure (Sahinkaya et al., 2007a)

2.4.3.5 Organic, sulfate and metal conversion rates and influent COD/SO_4^{2-} ratios

Generally, under mesophilic conditions, sulfate-reducing FBRs have been demonstrated to perform well under gradual intentional increase in sulfate and organic loading rates. Increasing the sulfate and the DOC loading rates up to 3500 and 900 mg/L·day, respectively, increments of sulfate reduction and electron donor removal percentages have been observed by Kaksonen et al. (2003a). FBRs respond well to changes in metal loading rates as well. With increasing Fe, Zn and Cd loading rates of 320, 220 and 20 mg/L·day, respectively, metal precipitation has been shown to be almost complete (Gallegos-Garcia et al., 2008).

As for HRT changes, temperature has been seen to affect the response of the reactors. Under thermophilic conditions, after an increase of sulfate and ethanol loading rates from 1000 to 1500 mg/L·day and from 320 to 485 mg/L·day, respectively, the process did not respond immediately (Sahinkaya et al., 2007a). The authors observed that the performance of the reactor initially decreased both in terms of sulfate reduction (down to 25%) and ethanol oxidation (down to 40%), but after just 10 days, the process recovered and a complete oxidation of ethanol and a 60% reduction of sulfate were observed. The sulfide concentration was enough to guarantee a complete precipitation of iron at an iron loading rate of 90 mg/L·day.

The robustness of a fluidized-bed sulfate-reducing process can be assessed also varying the COD/SO_4^{2-} ratio (Velasco et al., 2008). Under conditions of sulfate in excess (COD/SO_4^{2-} ratio of 0.11-0.20), using lactate and ethanol as carbon sources, Kaksonen et al. (2003a; 2003b) obtained an average sulfate reduction efficiency of 70% and the biogenic sulfide produced was enough to precipitate almost all the metals. According to Celis-Garcia et al. (2007), the optimal

COD/SO_4^{2-} ratio for sulfate reduction with lactate as electron donor was exactly the stoichiometric one (COD/SO_4^{2-} = 0.67 gram/gram). Increasing the sulfate loading rate from 1.5 up to 7.3 $gSO_4^{2-}/L \cdot day$ and keeping the organic loading rate stable, sulfate reduction became the main biological process occurring in the reactor with a strong enrichment of SRB in the biofilm.

In a different way, Villa-Gòmez et al. (2011) noticed that, in a IFBR fed with a COD/SO_4^{2} ratio of 5 (g/g), the COD removal efficiency was just around 30% due to the formation of acetate. Nevertheless, sulfate-reducing activity occurred at the highest conversion rates (2000 $mg/L \cdot day$ at a HRT of 9h), the sulfide production reached a value of 648 mg/L and 87% of the COD was consumed by the SRB.

2.4.3.6 Metal recovery in FBRs and IFBRs

FBRs and IFBRs are a single-stage process where sulfate-reducing activity and metal precipitation occur at the same time in the same reactor (Celis-Garcia et al., 2007). As described in the different sections above, in all the studies mentioned metal precipitated as sulfides almost totally and they can be found onto the surface of the biofilm, as fines in solution or settled to the bottom of the reactor (Kaksonen et al., 2003a; Villa-Gòmez et al., 2011). Villa-Gòmez et al. (2011) quantified the metals accumulated at the bottom of the reactors in their IFBR applications. The highest metal recovery percentages were 49.4%, 44.2%, 60.3% and 47.4% for Zn, Cu, Pb and Cd, respectively, but they could not recover the metals in a selective way. In fact, although IFBR characteristics allow metal precipitates to settle down, it is impossible to control the sulfide concentration as well as in a multiple-stage process (Tabak et al., 2003a).

2.4.3.7 Sulfide inhibition

As discussed in section 2.2.3.1, sulfide toxicity results are often contradictory. Also in fluidized-bed sulfate-reducing applications, the authors have found contrasting results about the sulfide toxic effects to SRB. Kaksonen et al. (2004b) carried out batch kinetic experiments by stopping the continuous flow in the FBR to study the effect of the dissolved sulfide on ethanol and acetate oxidation. Starting from a noncompetitive inhibition model (equation 17), the inhibition constants (k_I) for acetate and ethanol, added as electron donors, were obtained:

$$v = \frac{v_{max} \cdot S}{(k_m + S) \cdot (1 + \frac{I}{k_I})} \qquad (17)$$

(where v = oxidation velocity, v_{max} = maximum oxidation velocity, S = initial substrate concentration, k_m = Michaelis-Menten constant, I = inhibitor concentration and k_I = inhibition constant). H_2S inhibition constants for ethanol and acetate oxidation were 84 mgS-H_2S/L and 124 mgS-H_2S/L respectively, showing that ethanol oxidation is more affected by sulfide than acetate oxidation.

In contrast, Celis-Garcia et al. (2007) showed that, although the sulfide concentration reached a value of 1215 mg/L, both lactate consumption and sulfate reduction were not affected by the high sulfide production and kept stable around 90% and 75%, respectively. The high recirculation rate may have contributed to the formation of a biofilm able to tolerate high total sulfide concentrations without any apparent toxic effect.

2.4.3.8 Inhibition by heavy metals

High recycle ratios of FBRs allow treatment of wastewater with high concentrations of zinc (240 mg/L) and iron (58 mg/L) without inhibitory effects because of its dilution effect (Kaksonen et al., 2003a). In this way, SRB are exposed to concentrations much lower than the influent ones (Kaksonen et al., 2003b). Furthermore, the biogenic sulfide produced by the SRB quickly reacts with metals leading to the formation of metal sulfide particles less toxic and bioavailable for the bacteria (Isa et al., 1986a; 1986b).

The injection of zinc, iron and cadmium, added at maximum loading rates of 220, 320 and 20 mg/L·day, respectively, did not affect COD removal and sulfate reduction efficiencies in a sulfate-reducing IFBR (Gallegos-Garcia et al., 2008). Similarly, zinc, copper, lead and cadmium added each in a concentration of 10 mg/L, did not influence the sulfate-reducing activity of biomass grown in IFBRs of Villa-Gòmez et al. (2011).

On the contrary, Sahinkaya et al. (2007b) observed that metals can be toxic for SRB at 65°C even at very low concentrations. During the thermophilic FBR operation, the authors supplemented a trace element solution to the feed to overcome possible limitations in reactor performance. Unfortunately, this addition caused a quick decay in FBR efficiency since effluent acetate concentrations increased while sulfate reduction, dissolved sulfide concentration and effluent alkalinity decreased till the trace elements were excluded from the feed solution and the process slowly recovered.

Chapter 3 Materials and Methods

3.1 METAL PRECIPITATION EXPERIMENTS

The precipitation experiments were performed at room temperature (23 ± 2°C) in order to assess the depletion kinetics of Zn, Cu, Pb and Cd from a multi-metal system. 117-mL serum bottles (Figure 9) were used containing synthetic wastewater only (Table 13) or 112 mL of synthetic wastewater and 5 mL of biofilm-coated polyethylene beads. Polyethylene beads (3 mm diameter) covered with a SRB biofilm were collected at the end of an IFBR operation run as described by Villa-Gòmez et al. (2011). The pH of synthetic wastewater was adjusted to 7 to simulate the optimal biological sulfate-reducing conditions.

Table 13- Composition of the synthetic wastewater used for the metal precipitation experiments

Compound	Concentration (mg/L)
Macronutrients	
$FeSO_4 \cdot 7H_2O$	50
$CaCl_2 \cdot 2H_2O$	2500
NH_4Cl	200
KH_2PO_4	500
$MgSO_4 \cdot 7H_2O$	2500
Metals	
Zn^{2+}	10
Cu^{2+}	10
Pb^{2+}	10
Cd^{2+}	10

Two sets of experiments were carried out at sulfide concentrations of 0, 20, 40 and 80 mg/L using $Na_2S \cdot 9H_2O$ as sulfide source. These sulfide concentrations were chosen to simulate bioreactors operating above and below the stoichiometric amounts required for metal sulfide precipitation. The first precipitation experiment was done with synthetic wastewater only (S1, S2, S3

and S4), whilst the second one with biofilm coated polyethylene beads (B1, B2, B3, B4). The bottles were shaken at a rate of 100 rpm throughout the experiment. Metal depletion was followed by taking samples of the liquid phase every 3h during the first 9h and from 24h to 30h.

Figure 9 - Serum bottles used for metal depletion batch experiments

3.1.1 Analytical methods

Metal measurements were done by flame spectroscopy (AAS 3110, Perkin Elmer, USA) and furnace spectroscopy (AAS Solaar MQZe GF95, Perkin Elmer, USA) after dilution, acidification with HNO_3 and filtration of the samples. The precipitates of the experiments S1, S2, S3 and S4 were collected and centrifuged at 5000 rpm during 10 min for X-ray absorption near edge spectroscopy analysis (XANES). XANES was performed on Zn precipitates on the DUBBLE beam line BM26A of the European Synchrotron Radiation

Facility (Grenoble, France) (Borsboom et al., 1998). Spectra for Zn species were collected from multiple reference compounds, including ZnS, Zn sorbed on apatite and $Zn_3(PO_4)_2 \cdot 4H_2O$. The X-ray energy ranged from 200eV below and 750eV above the adsorption K-edge of Zn (9659eV).

3.1.2 Data analysis

The kinetic parameters of Zn, Pb and Cd were determined by monitoring the depletion of the metal concentration through time. The rate constants for metal precipitation were determined using a first order equation:

$$\frac{dM}{dt} = -k \cdot t \qquad (18)$$

The value of the kinetic constants was evaluated by plotting the logarithm of the concentration versus time (equation 19) (Brezonik, 1994):

$$\ln M = \ln M_0 - k \cdot t \qquad (19)$$

where "M" is the metal concentration at a certain time, "M_0" is the initial metal concentration and "k" is the metal depletion rate constant. A plot of "ln M" versus time results in a straight line for reactions following first-order kinetics with "k" obtained from the slope of the line.

3.2 SULFATE-REDUCING BIOREACTORS

Two Plexiglas IFBRs (volume 5.7 L, height 1.13 m and inner diameter 0.08 m) were used to enrich and maintain SRB at room temperature, each using an external device as water level adjustor (Figure 10 and Figure 11).

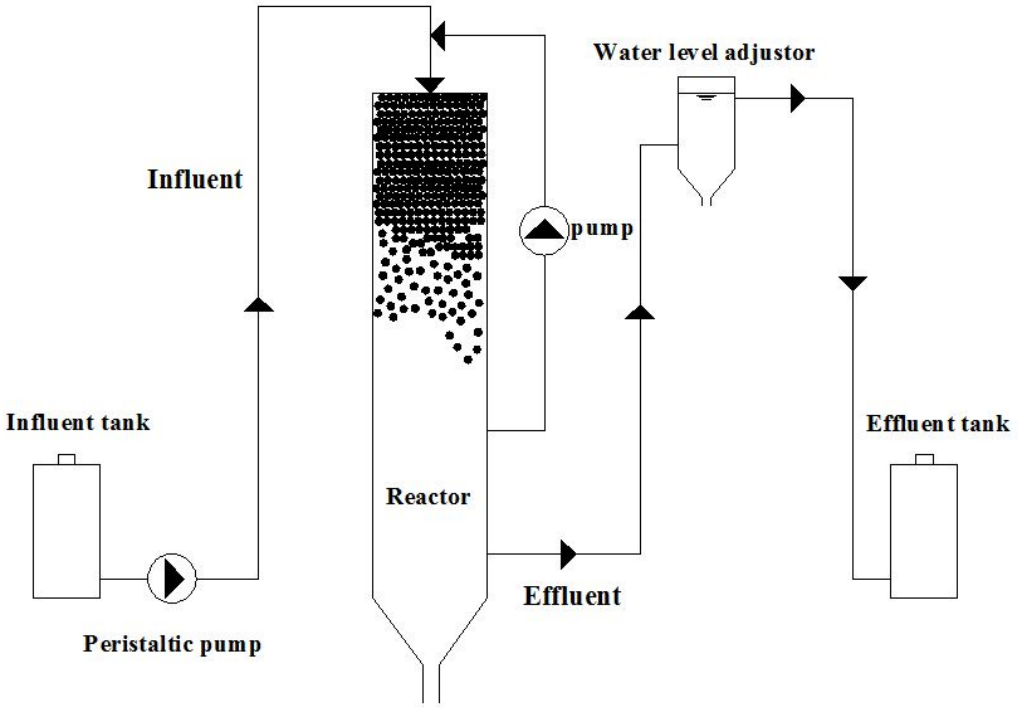

Figure 10 – Experimental setup of the IFBRs

Polypropylene pellets (volume = 500 mL, particle size = 3-5 mm) were used as carrier material in both reactors. The recirculation flow was set to guarantee a 10% fluidization degree (i.e. 10% increase of the bed height) and a superficial velocity of 18 m/h. Each reactor was inoculated with 50 mL of methanogenic granular sludge (20 gVSS/L) from a full-scale anaerobic digester fed with buffalo manure and dairy wastewater.

Figure 11 - Experimental sulfate-reducing IFBRs

3.2.1 IFBR operational conditions

The two IFBRs were operated for 242 days at room temperature (22-25°C). Before the introduction of the support material in the reactors, the polypropylene pellets (Figure 12) were first crashed with silica sand to increase their surface area for a better biomass attachment.

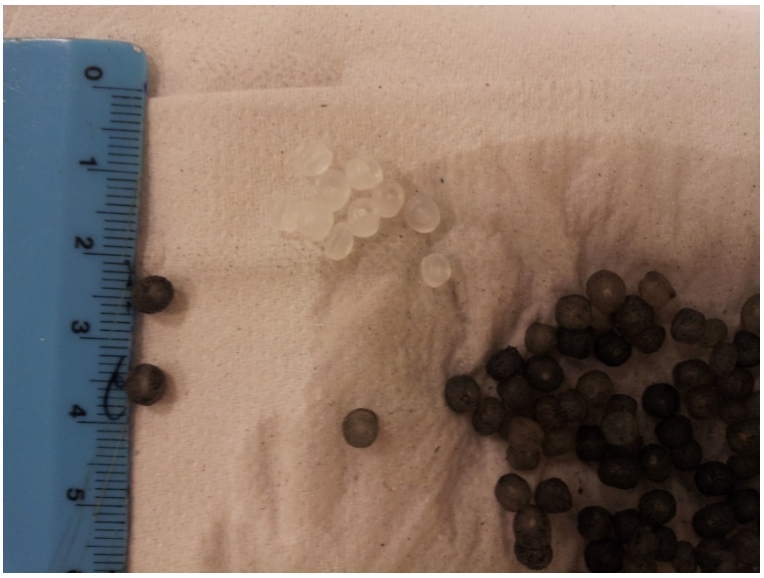

Figure 12 - Polypropylene pellets used during the sulfate-reducing experimentation. The white pellets are the unused ones; the brown pellets are the biomass-colonized ones

A synthetic medium containing lactate, sulfate and nutrients (Table 14 and Table 15) was fed to the reactors at a HRT of 24 h. After inoculation, both reactors were started-up for 35 days (Period I) to allow the colonization of the polypropylene pellets with a COD/SO_4^{2-} (g/g) ratio of 0.67. This ratio is the theoretical one according to the stoichiometry (reaction 1) and it was shown to be the optimal one to stimulate the sulfate-reducing activity over other fermentative kinetics (Celis-Garcia et al., 2007).

Table 14- Composition (mg/L except for pH) of the IFBRs feed solution

Compound	**IFBR 1**	**IFBR 2**
Lactic acid (in terms of COD)	1000	1000 → 4000
Sulfate (fed as Na_2SO_4)	1500	1500 → 1000
KH_2PO_4	200	200
NH_4Cl	300	300

KCl	250	250
$MgCl_2 \cdot 6H_2O$	120	120
$CaCl_2 \cdot 2H_2O$	15	15
Trace metals (Table 2)	yes	yes
pH	4.61 → 7.00	3.00 → 7.00

Table 15 - Composition of the trace metal solution used as micronutrient for feeding the two sulfate-reducing IFBRs

Compound	Concentration (mg/L)
$FeCl_2 \cdot 4H_2O$	1500
$MnCl_2 \cdot 6H_2O$	118
$AlCl_3 \cdot 6H_2O$	40
$Na_2MoO_4 \cdot 2H_2O$	36
$NiCl_3 \cdot 6H_2O$	24
$CoCl_2 \cdot 6H_2O$	70
HCl 36%	1 mL

*20 mL of this solution were diluted in 20 L of IFBR feed solution

In this period the pH was adjusted to 7 and the reactors were operated in batch mode to let SRB grow faster. Subsequently, the reactors were operated under other five operating conditions (summarized in Table 16) in order to test the effect of COD/SO_4^{2-} ratio and feed pH on the reactor performances.

Table 16 - Operational conditions for the two sulfate-reducing IFBRs

Parameter	**Experimental periods**					
	I	**II**	**III**	**IV**	**V**	**VI**
Days	0-35	36-49	50-157	158-182	183-195	196-242
IFBR 1						
Influent COD (mg/L)	1000	1000	1000	1000	1000	1000
Influent SO_4^{2-} (mg/L)	1500	1500	1500	1500	1500	1500
COD/SO_4^{2-} ratio	0.67	0.67	0.67	0.67	0.67	0.67

pH *	7.00	5.29 ± 0.28	5.24 ± 0.15	4.84 ± 0.11	5.01 ± 0.03	4.86 ± 0.18
HRT (day)	1	1	1	1	1	1
IFBR 2						
Influent COD (mg/L)	1000	4000	4000	4000	3000	3000
Influent SO_4^{2-} (mg/L)	1500	1000	1000	1000	1000	1000
COD/SO_4^{2-} ratio	0.67	4.00	4.00	4.00	3.00	3.00
pH *	7.00	3.05 ± 0.05	5.31 ± 0.16	3.09 ± 0.04	3.28 ± 0.02	5.05 ± 0.08
HRT (day)	1	1	1	1	1	1

* pH expressed as mean value ± standard deviation

3.2.2 Calculations

COD removal and sulfate reduction efficiencies are defined as:

$$\text{COD removal efficiency (\%)} = \frac{COD_{in} - COD_{out}}{COD_{in}} \cdot 100 \qquad (20)$$

$$SO_4^{2-} \text{ reduction efficiency (\%)} = \frac{SO_4^{2-}{}_{in} - SO_4^{2-}{}_{out}}{SO_4^{2-}{}_{in}} \cdot 100 \qquad (21)$$

where COD_{in}, $SO_4^{2-}{}_{in}$ and COD_{out}, $SO_4^{2-}{}_{out}$ are the COD measure and sulfate concentration in the feed and in the outlet, respectively.

In order to evaluate the development of the sulfate reduction process in the bioreactors, for each experimental period the mean percentage of COD used by SRB has been calculated as follows:

$$\text{COD to sulfate (\%)} = \frac{0.67 \cdot (SO_4^{2-}{}_{in} - SO_4^{2-}{}_{out})}{COD_{in} - COD_{out}} \qquad (22)$$

where 0.67 is the theoretical COD/SO_4^{2-} ratio according to reaction (1).

The residual COD degradation has been supposed to be due to biomass growth and other fermentative reactions.

The effluent acetate concentration has been expressed as COD according to reaction (23) and its percentage in the effluent COD has been calculated as shown in equation (24):

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O \quad (23)$$

$$\text{Acetate in the effluent COD (\%)} = \frac{CH_3COOH_{asCOD}}{COD_{out}} \cdot 100 \quad (24)$$

3.2.3 Physico-chemical analyses

Samples for sulfate, COD, acetate, lactate, sulfide and pH were taken and analyzed twice a week. Sulfate and COD were analyzed from the beginning of the reactor operation. Acetate, lactate and effluent pH were analyzed from day 36 and sulfide from day 45 to day 206. Sulfate, COD and pH were measured in both influent and effluent solutions, acetate and lactate were measured only in the effluent solution while sulfide samples were taken from the water level adjustor. For sulfate, COD, acetate and lactate analysis, the samples were filtered using 0,45 µm membrane filters (Millipore, USA). Sulfate was measured by ion chromatography by using a 883 Basic IC Plus (Metrohm, Switzerland). COD was determined by the Closed Reflux Method by using a CR2200 digester and a PholoLab S6 photometer (WTW, Germany). Sulfide was measured with a UV-Lambda10 spectrophometer (Perkin Elmer, USA) (Cord-Ruwisch, 1985) and the samples were collected in plastic vessels, filled up to the edge and closed to avoid any loss. Acetate and lactate were determined using a

series 200 HPLC equipped with a Pinnacle ODS 250x4.6mm column (Restek, USA) and a LC 905 spectrophotometric UV detector (Perkin Elmer, USA). Liquid pH was monitored by a Pt-100 electrode (Crison, Spain).

3.3 NITRATE-REMOVING APPLICATIONS

3.3.1 Nitrate-reducing FBRs

Three glass FBRs (volume 1.1 L) were used to enrich and maintain denitrifying bacterial cultures (Figure 13). Granular activated carbon (volume = 200 mL, particle size = 0.5-1 mm) was used as biomass carrier.

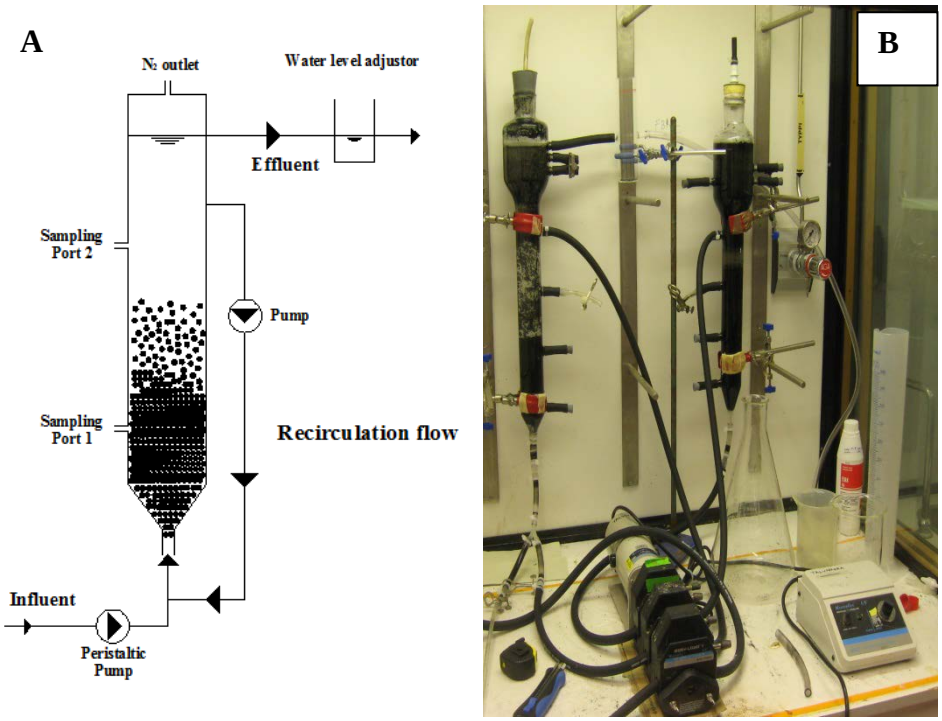

Figure 13 - Nitrate-removing FBRs: configuration (A) and photograph (B)

The recirculation resulted in a 25% carrier fluidization. FBRs were seeded with 240 mL of activated sludge (2.53 g VSS/L) from municipal wastewater treatment plant in Helsinki, Finland.

3.3.2 FBR operational conditions

The three FBRs were operated for 368 days. Reactor 1 (FBR1) was operated at 7-8°C (Figure 14), and FBR2 and FBR3 were operated at 22 ± 2°C (Figure 13B). FBR1 and FBR2 were used for studying process performance, while FBR3 was used for biomass enrichment for batch assays.

Figure 14 - Nitrate-reducing FBR1 operated under psychrophilic conditions and placed in a fridge

Table 17 - Composition of the nitrate-removing FBR solution

Compound	Concentration (mg/L)
KH_2PO_4	50
$CaCl_2 \cdot 2H_2O$	20
$MgCl_2 \cdot 6H_2O$	150
$Na_2MoO_4 \cdot 2H_2O$	0.1
$MnCl_2 \cdot 4H_2O$	1.75
$CoCl_2 \cdot 6H_2O$	0.05

After seeding, a medium containing ethanol, nitrate and nutrients (Table 17) was flushed with nitrogen and fed to the FBRs. At first, the reactors were operated for 43 days (Period I) to allow the bacterial colonization of the granular activated carbon particles using a nitrate concentration of 186 mg/L with a C/N (mol/mol) ratio of 2.5 (Heylen et al., 2006). During this period the pH was adjusted to 7.5 and the reactors were operated in batch mode. Half of the FBR volume was replaced with fresh medium twice per week. After Period I, the FBRs were operated in continuous mode under twelve operating experimental periods (summarized in Table 18) in order to test the effects of the temperature, HRT and the feed pH on process performances. The process was monitored by sampling both the liquid phase and biomass from the two sampling ports (Figure 13A).

Table 18 - Operational conditions for nitrate-removing FBR1 and FBR2*

Parameter	**Experimental periods**												
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**
Days	0-43	44-123	124-165	166-179	180-197	198-211	212-225	226-239	240-256	257-295	296-309	310-	
Ethanol (mg/L)	172.5	62.0	124.0	124.0	124.0	124.0	124.0	124.0	124.0	124.0	124.0	124.0	124.0
Nitrate (mg/L)	186.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Ethanol/NO_3^- (mol/mol)	1.25	0.42	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83
pH	7.50	7.00	7.00	7.00	6.00	5.50	5.00	4.50	4.00	3.50	3.00	2.80	2.50
HRT (h)	batch	9	9	6	6	6	6	6	6	5.4	5.4	5.4	5.4

* FBR1 and FBR2 were operated at 7-8°C and room temperature, respectively, throughout the experiment.

3.3.3 Batch assays

Batch experiments to assess the effect of acidic pH and the ethanol/nitrate ratio on the bacterial activity were performed at room temperature (22°C) in serum bottles of 117 mL in a gyratory shaker (200 rpm) (Figure 15). The bottles contained 5 mL of biomass on carrier material from FBR3 and 110 mL of medium (Table 19). The pH of the solution was adjusted to the desired value using HCl and the bottles were flushed with nitrogen. The experiments were carried out using a stoichiometric ethanol/nitrate ratio (0.42:1 mol/mol).

Table 19 - Composition of the bottles used for the batch tests

	Feed pH	**Ethanol (mg/L)**	**Nitrate (mg/L)**	**Nutrient solution (mL)**	**Biomass (mL)**	**Ethanol re-spiking (mg/L) after 4.5 h**
Bottles						
Bottle 1	3.0	62	200	110	5	-
Bottle 2	3.5	62	200	110	5	-
Bottle 3	4.0	62	200	110	5	-
Bottle 4	4.5	62	200	110	5	-
Bottle 5	4.0	62	-	110	5	-
Bottle 6	5.0	62	-	110	5	-
Bottle 7	4.0	62	200	110	5	62
Bottle 8	5.0	62	200	110	5	62

The denitrification process was monitored by measuring ethanol, nitrate, nitrite and pH from the samples taken every 1.5 hours interval for a total duration of 9 hours. In order to evaluate the possible competition between different microbial species and to better assess the degradation of the electron donor throughout the experiment, “control bottles” were run without nitrate in solution or spiking the solution with ethanol again after 4.5 hours from the beginning of the experiment (Table 19).

Figure 15- Serum bottles used for carrying the batch experiments out

3.3.4 Physico-chemical analyses

Total suspended solids (TSS) and volatile suspended solids (VSS) in the original inoculum were determined according to standard methods (APHA, 2005). Nitrate, ethanol, nitrite, pH were analyzed twice a week from the beginning of the reactor operation. Samples for nitrate, nitrite and ethanol analyses were filtered using 0,45 µm membrane filters (Whatman, UK). Nitrate and nitrite were measured by ion chromatography by means of a IC DX-120 equipped with a 4x250 mm column IonPac As23 (Dionex, Thermo Fisher, USA). Ethanol was determined by using a LC-20AC prominence HPLC equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and a 30-cm Rezex RHM Monosaccharide H+ (8%) column (Phenomenex, Alleroed, Denmark). 0.01 N H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml/min. Liquid pH was controlled and monitored by a SenTix 41 pH-electrode (WTW, Germany).

3.3.5 Microbiological analyses

The microbial communities in the nitrate-reducing FBRs were studied three times during the reaction operation using the PCR/DGGE procedure (Figure 16). Each time 20 ml of reactor liquor was filtered on 0.2 µm polysulfone membranes (Whatman, UK). Total DNA was extracted from filters using the Ultraclean Soil DNA Extraction Kit (MoBio Laboratories, USA). PCR was carried out as described by Kolehmainen et al. (2007) using primers Ba357F-GC and Un907R. Denaturing gradient gel electrophoresis (DGGE) was performed for analyzing bacterial communities according to Kolehmainen et al. (2007) having a denaturing gradient from 30% to 70 %. Gel was run at 60 °C in 1 × TAE with 100 V for 20 h and stained SYBR® Gold (Molecular Probes, Inc., Eugene, OR). The DGGE bands were excised from the gel and re-amplified for sequencing using forward primer had no GC-clamp. Sequencing was performed by MacroGen (Seoul, Korea). Sequence data was identified via comparison with the database of the National Center for Biotechnology Information (NCBI) using MegaBLAST (Zhang et al., 2004).

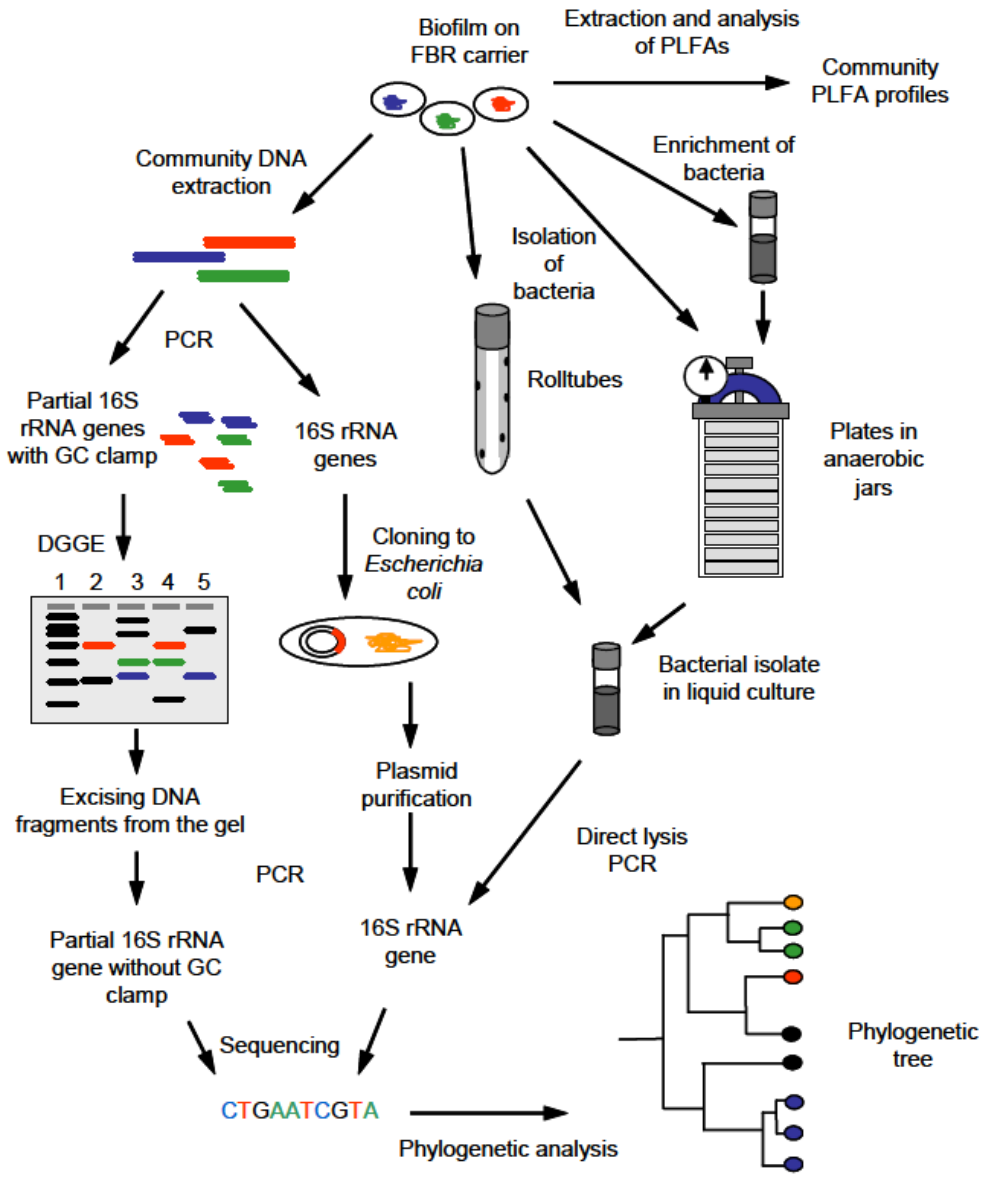

Figure 16 – Analysis of bacterial diversity by denaturing gradient gel electrophoresis (DGGE) and cloning of polymerase chain reaction (PCR) – amplified 16S rRNA genes (figure by Kaksonen, 2004c).

Chapter 4

Results

4.1 METAL PRECIPITATION EXPERIMENTS

4.1.1 Metal depletion kinetics

The reaction between metal and sulfide in the batch experiments was almost instantaneous, resulting in the formation of a brownish solution. Figure 17 and Figure 18 show the metal depletion rates at different sulfide concentrations for the experiments with synthetic wastewater only (S1, S2, S3 and S4) and a biofilm-coated polyethylene support (B1, B2, B3 and B4).

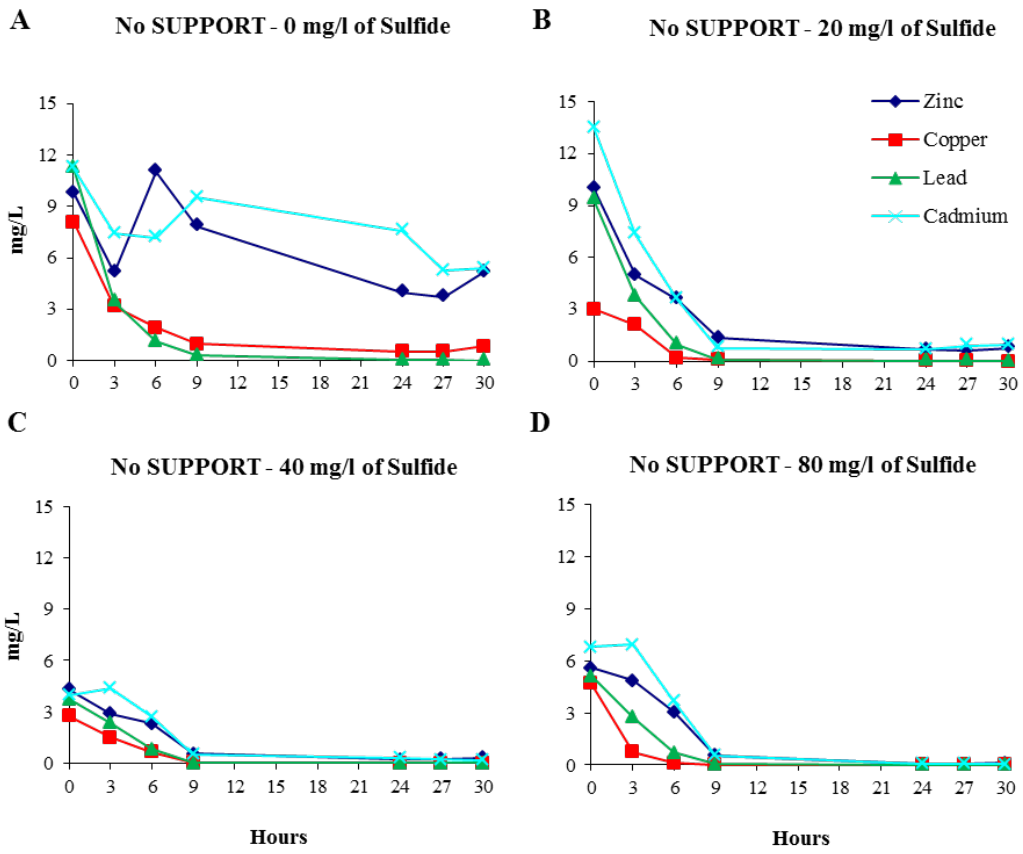

Figure 17 - Metal concentration in the liquid phase from batch experiments with synthetic wastewater only at 0 (A), 20 (B), 40 (C) and 80 (D) mg/L of sulfide

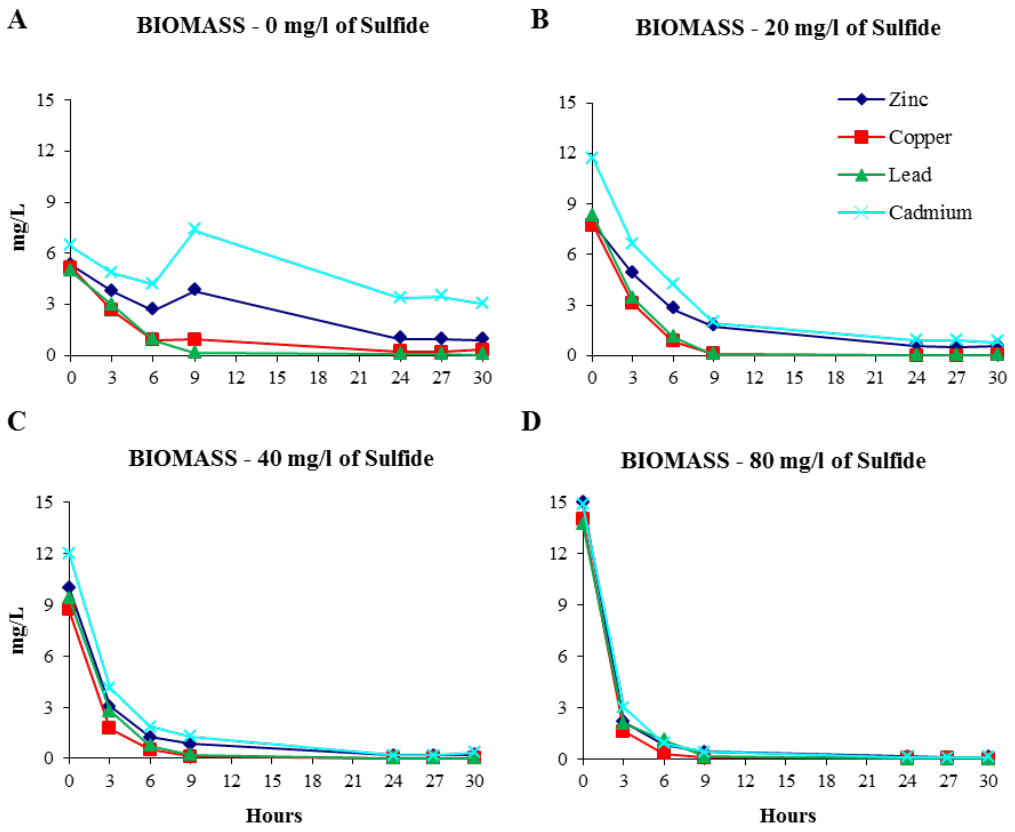

Figure 18 - Metal concentration in the liquid phase from batch experiments with a biofilm-coated polyethylene support at 0 (A), 20 (B), 40 (C) and 80 (D) mg/L of sulfide

Metals precipitated and reached equilibrium concentration within 9h. Increasing sulfide concentration, Zn and Cd precipitated quickly such as Pb and Cu. Figure 17 shows that Cu was removed mainly in the first 6 hours when sulfide was in solution. Moreover, its initial concentration was clearly below 10 mg/L because of supersaturation phenomena after sulfide dosing. Zn and Cd depletion was different in batch experiment without sulfide addition (Figure 17A and Figure 18A). In experiment S1, 6 mg/l of Zn remained in the liquid phase, whereas 3 mg/L of Zn remained in B1. For Cd, 6 mg/L remained in the S1 liquid phase after 30 h while 0.9 mg/L remained in the liquid phase for B1. Batch

experiments where sulfide was added showed no significant difference in metal depletion rates suggesting that the biofilm did not influence metal removal in the presence of sulfide.

4.1.2 Kinetic parameters

S2, S3 and S4 results were considered for the evaluation of the kinetic parameters (Figure 19).

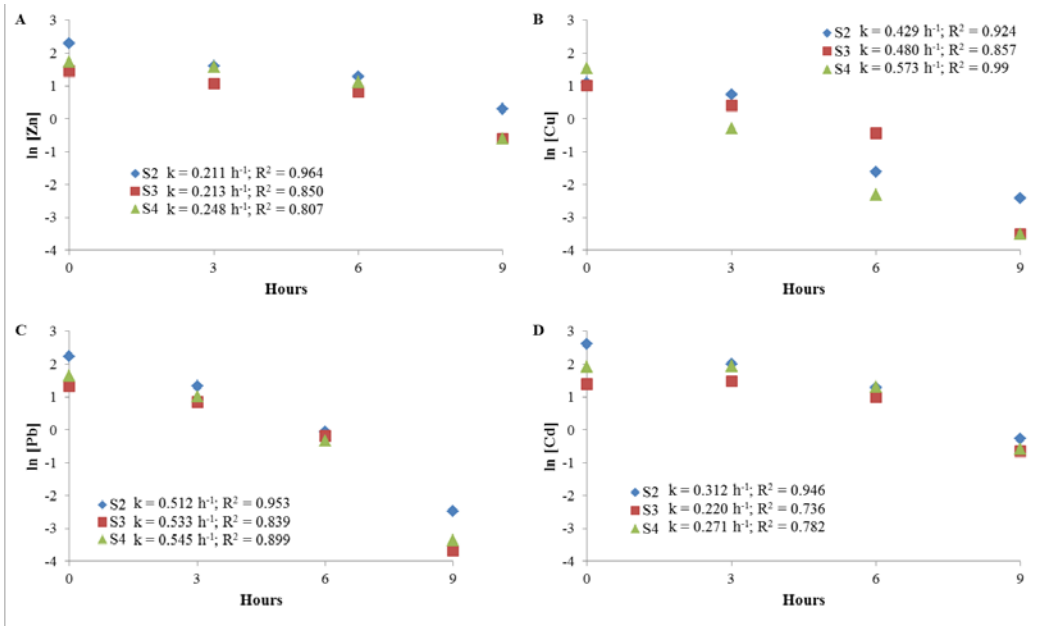

Figure 19 - Ln [M] vs. time of Zn (A), Cu (B), Pb (C) and Cd (D) for experiments S2, S3 and S4

All the metals showed a similar rate for the three experiments reaching the equilibrium after 9h in the following order: Cu > Pb > Cd > Zn. Cu depletion was very fast resulting in an almost complete removal of copper after 6h. For Cu depletion rate kinetic calculation, the only result from S4 was taken into account

since experiments S2 and S3 has relatively significant deviations. The kinetic constants for Pb showed no significant differences for S2, S3 and S4. Zn and Cd more significant depletion rate constants were $0.211h^{-1}$ and $0.312\ h^{-1}$, respectively, showing that Zn and Cd removal kinetics were the slowest ones.

4.1.3 Solid phase characterization

Of the four metals tested, Zn has the high solubility product and showed to have the lowest depletion rate constant. As shown in Figure 17A and Figure 18A, Zn was more likely removed by other removal mechanisms. Therefore, XANES analysis was performed to examine the Zn speciation in the experiments (Figure 20).

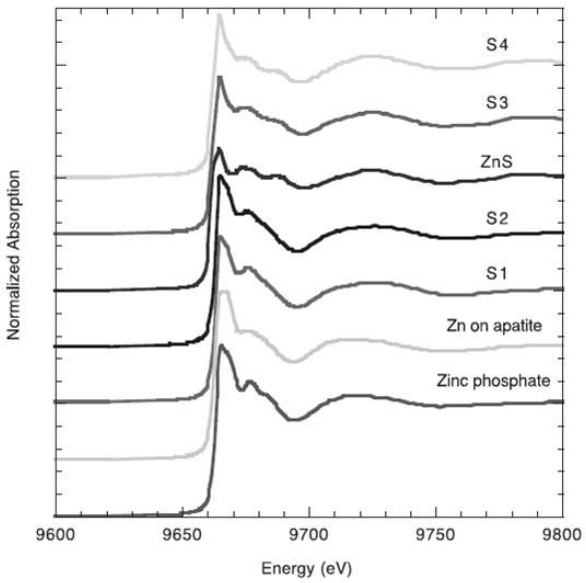

Figure 20 - Comparison of the XANES spectra for the batch experiments S1, S2, S3 and S4, and the reference compounds ZnS, $Zn_3(PO_4)_2 \cdot 4H_2O$ and Zn sorbed on apatite

The spectra of the four experiments fit with only three model compounds: ZnS, Zn sorbed on apatite and $Zn_3(PO_4)_2 \cdot 4H_2O$. The spectra for the experiments with the highest sulfide concentrations (S3 and S4) displayed high similarities with the ZnS XANES spectra implying that Zn was mainly removed as sulfide salt. In contrast, experiments with the lowest sulfide concentrations displayed a better fitting with the Zn sorbed on apatite and Zn phosphate spectra, demonstrating that such phenomena are involved in Zn removal at low sulfide concentrations.

4.2 SULFATE-REDUCING BIOREACTORS

Figure 21 compares the feed and effluent characteristics of the two reactors in terms of COD removal and sulfate reduction:

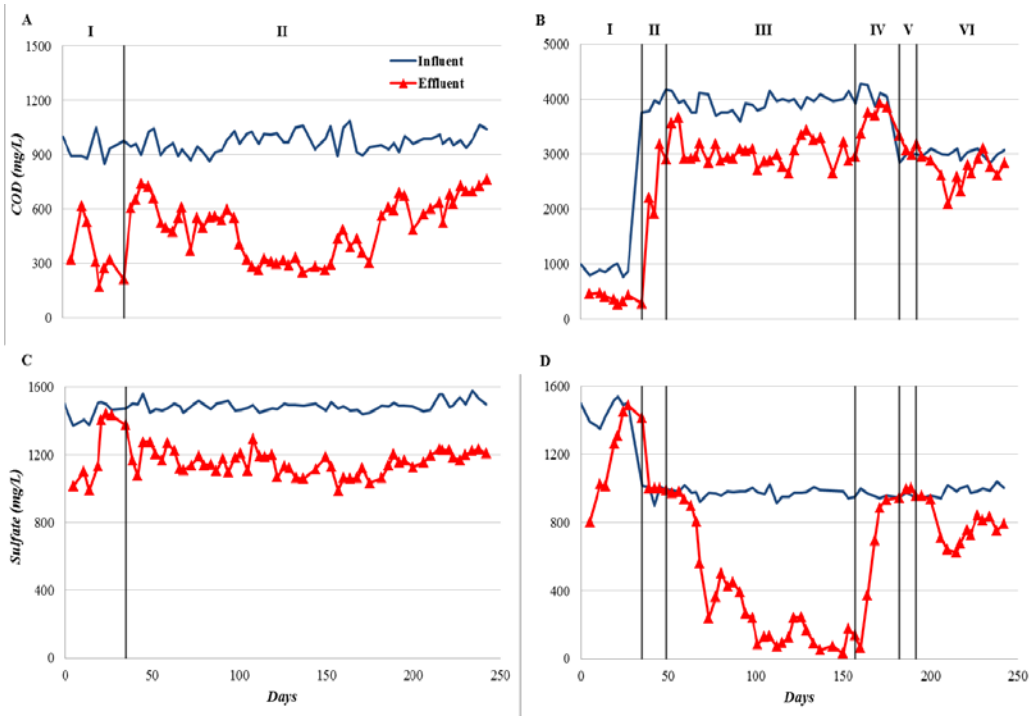

Figure 21 - Influent and effluent COD and sulfate in IFBR1 (A,C) and IFBR2 (B,D)

Using lactate as electron donor, a period of about 35 days was necessary to obtain an appreciable colonization of the polypropylene pellets by the SRB operating the reactors in batch mode. In this period, because of the enrichment of biomass, the COD removal efficiencies were the highest reaching 83% and 74% in IFBR1 and IFBR2, respectively. However, the performances of both reactors were characterized by instabilities in COD and sulfate removal.

A stoichiometric COD/SO_4^{2-} ratio of 0.67 was used to carry out the start-up phase in both reactors. Low sulfate reduction efficiencies were observed during this experimental period (i.e. average values of 19% and 20% in IFBR1 and IFBR2, respectively) and only 35% of the COD degraded was used for sulfate reduction in both reactors. This was probably due to the heterogeneous seed used as source of biomass. SRB showed a slower adaptation to the system than other active microorganisms in the biofilm (Kalyuzhnyi et al., 1998; D'Acunto et al., 2011; Frunzo et al., 2012).

With the decrease of the feed pH in period II from 7 to 5 and from 7 to 3 (Figure 22) in IFBR1 and IFBR2, respectively, the COD removal and the sulfate reduction efficiencies further decreased in both reactors. In IFBR1, COD and sulfate removal percentages were on average 18% and 9%, respectively, whereas in IFBR2 the SRB activity resulted almost completely inhibited as the sulfate reduction efficiency was just 1% and the effluent pH dropped to 3.

From day 49 to day 181 (experimental periods III and IV), the performance of IFBR1 was stable and COD and sulfate removal efficiencies remained on average values of 59% and 26%, respectively. Effluent pH (Figure 22A and B) and sulfide (Figure 24) trends were constant as well and stable to mean values of 7.28 and 108 mgS/L, respectively. During this experimental period, the highest sulfate reduction rate was 525 mg/L·day on day 157 resulting in a sulfate reduction efficiency of 35%. The COD removed by SRB slightly increased up to

61% on day 192, and thus the sulfate reduction became the predominant process in the system. Nevertheless, the presence of other microbial groups and the effect of the competition for lactate is well shown in Figure 21A. From day 98 to day 182, an increase of the COD consumption from 43% to 66% was observed without any increase of the sulfate reduction. Moreover, acetate accumulation in concentration of 200 mg/L in IFBR1 contributed to the low sulfate reduction efficiencies (Figure 23). Lactate always remained below the detection limit, indicating an incomplete conversion of lactate to acetate and stopping the sulfate reduction at 35% of sulfate removal.

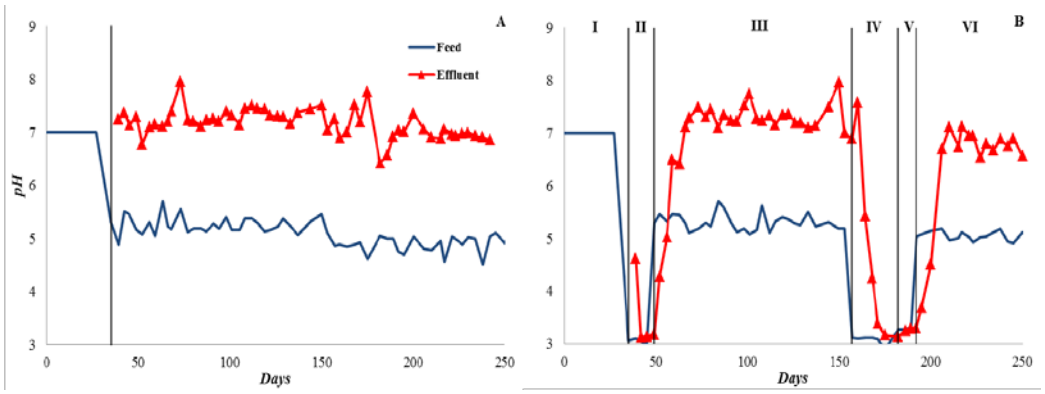

Figure 22 - Evolution of feed and effluent pH in IFBR1 (A) and IFBR2 (B)

On the contrary, during period III, IFBR2 showed higher sulfate reduction yields, once the feed pH was increased to 5 again. The excess of lactate over sulfate continuously guaranteed the required carbon source for the SRB to reduce sulfate to sulfide. This resulted in a 97% of sulfate removal efficiency with a COD/SO_4^{2-} ratio of 4 after a complete recovery from the previous failure period. Sulfide concentration and effluent pH increased very quickly up to 310 mgS/L and 8, respectively. The average COD removal efficiency was 24% and

the mean percentage of COD to sulfate reduction was 45%. Because of the incomplete oxidation of lactate, acetate accumulated in IFBR2 solution up to 2322 mg/L, whereas lactate was below the detection limit (Figure 23). The highest acetate concentrations corresponded to the highest performances of the system in terms of sulfate reduction, but acetate accumulation did not affect the sulfate reduction process in IFBR2 because of the excess of lactate in solution. In order to test IFBR2 again with a sudden feed pH change from 5 to 3, during period IV and V, the process failed. The mean sulfate reduction efficiency decreased first to 49% (period IV) and then to 2% (period V) resulting in a complete SRB inhibition. The effluent pH and COD removal efficiency dropped as well to 3 and 6%, respectively. As a sulfate reduction product, acetate was present in solution at lower concentrations, on average equal to 132 mg/L. Consequently, lactate concentration in the reactor increased to 3050 mg/L.

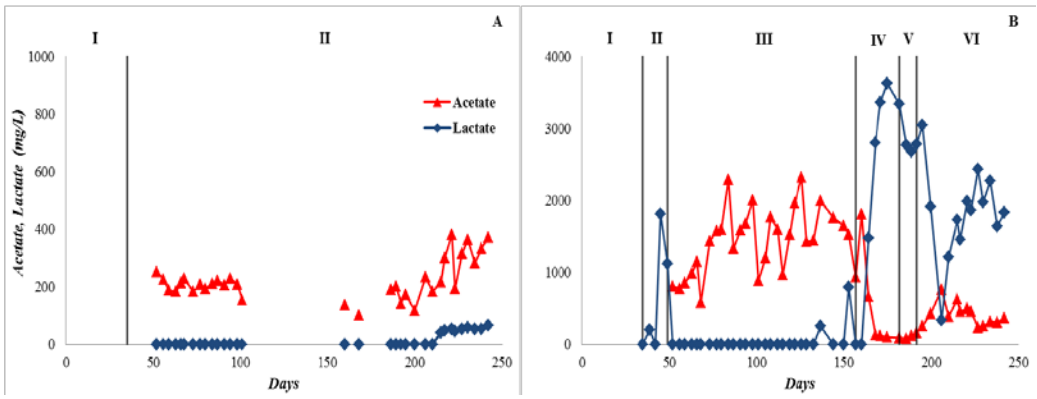

Figure 23 - Effluent acetate and lactate in IFBR1 (A) and IFBR2 (B)

After the accidental emptying of IFBR1, in periods V and VI , COD and sulfate removal efficiencies decreased to 38% and 22%, respectively. Acetate was detected at a concentration of about 250 mg/L, limiting the development of the

sulfate-reducing activity. Sulfide concentration and effluent pH slightly decreased to 103 mg/L and 6.95, respectively.

In period VI, the process partially recovered in IFBR2 because of the increase of the feed pH to 5 with a COD/SO_4^{2-} ratio of 3. COD and sulfate removal efficiencies increased up to 23% and 37% as peak values, respectively. As a result, acetate concentration increased again up to 766 mg/L and influent pH was completely neutralized.

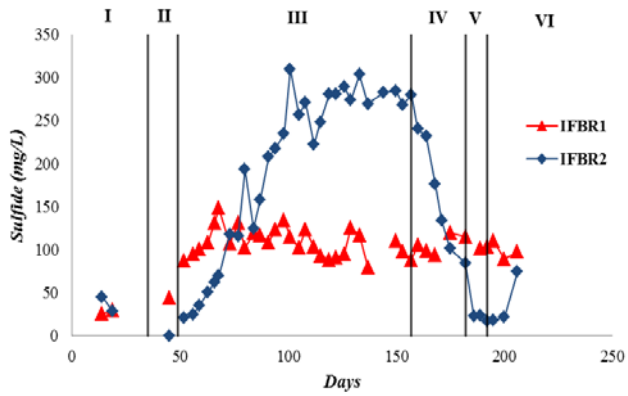

Figure 24 - Sulfide production in IFBR1 and IFBR2

4.3 NITRATE-REMOVING APPLICATIONS

4.3.1 Batch assays

Figure 25 shows the temporal profiles of nitrate, ethanol, pH and nitrite at different feed pH conditions in batch assays. Nitrate and ethanol removals and nitrite accumulation were similar at initial pH's of 3.5-4.5 and were inhibited at pH 3. Under these conditions, the nitrate concentration in the solution was found to be 106 mg/L after 9 hours from the beginning of the experiment. Ethanol was totally removed at initial pH's of 3.5-4.5, whilst ethanol removal only reached

38% at feed pH of 3.0, although an initial quick decrease within the first 1.5 hours. In all batch assays the pH increased rapidly during incubation. When this pH increase is accounted for, the actual inhibitory pH was approximately 4.8.

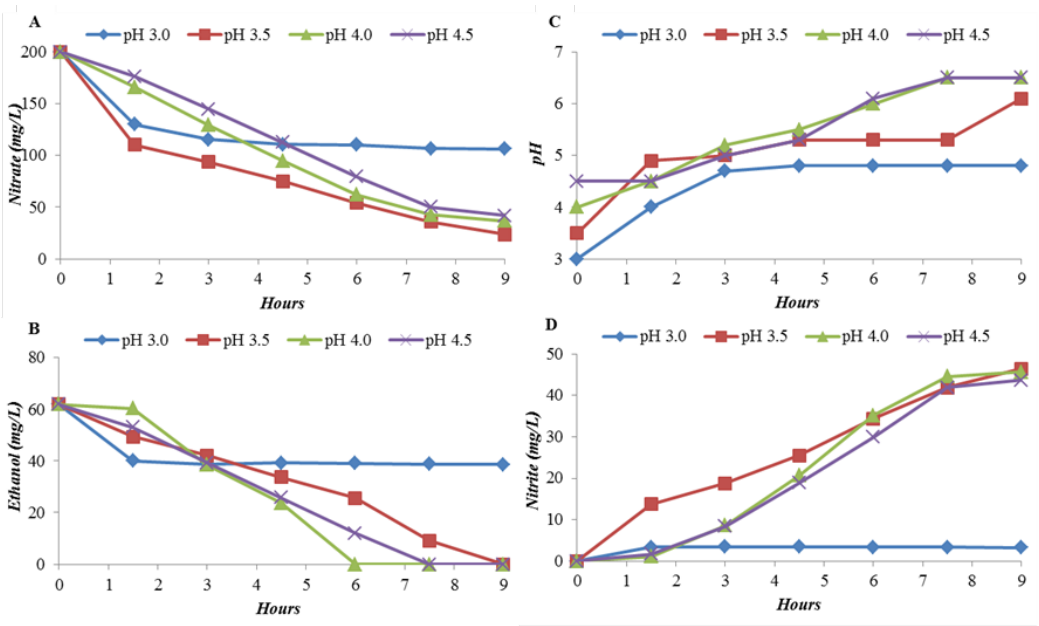

Figure 25 – Effect of pH on nitrite (A) and ethanol (B) removals, pH (C) and nitrite (D) accumulation in batch assays

For a better evaluation of the possible competition between different microbial species and the ethanol degradation kinetics, a second experiment was carried out using “control bottles”. Two bottles (bottles 5 and 6) were fed with no nitrate. The other two bottles (bottles 7 and 8) were re-spiked with ethanol after 4.5 hours from the beginning of the experiment. In Figure 26 the results from this batch test are reported:

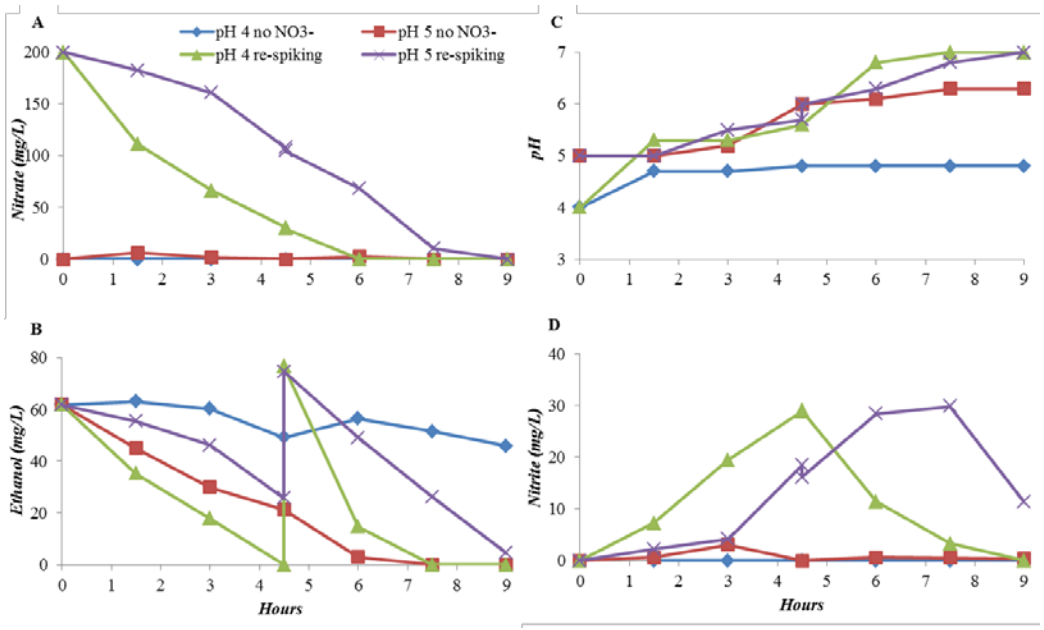

Figure 26 - Nitrate (A), ethanol (B), pH (C) and nitrite (D) trends in bottles 5 and 6 (fed without nitrate) and bottles 7 and 8 (re-spiked with ethanol after 4.5 hours)

Bottles 5 and 6 showed lower ethanol removal percentages than in the previous test. In bottle 5, fed with a pH of 4.0, ethanol concentration was 46 mg/L after 9 hours resulting in a 26% of ethanol removal. However, ethanol was completely consumed within 7.5 hours in bottle 6 at feed pH of 5.0. The initial pH of 4.0 was not increased in bottle 5, whereas in bottle 6 the pH was raised to 6.3 starting from a value of 5.0.

At the beginning, bottles 7 and 8 were fed with ethanol and nitrate in stoichiometric conditions. To evaluate the effect of the ethanol/nitrate ratio on the biological process, the two bottles were re-spiked with ethanol after 4.5 hours. Nitrate was completely removed within 6 and 7.5 hours, in bottles 7 and 8, respectively. Although injected twice, ethanol remained below the detection limit at the end of the experiment. The feed pH was neutralized in both bottles.

Nitrite concentration first increased up to 30 mg/L and then decreased with the ethanol re-spiking.

4.3.2 Fluidized-bed reactor performances

Removal of nitrate from the synthetic medium was investigated using enriched denitrifying cultures on ethanol in FBR1 and FBR2 run for 368 days under different operational conditions (Figure 27A). Figure 27 (B-C) shows FBR performances, i.e. ethanol and nitrate removals and the pH evolution at 7-8°C and 22°C. Denitrifying bacteria showed to immediately adapt to the environmental conditions both at 7-8°C and 22°C. High removal efficiencies were achieved as effluent nitrate and ethanol remained below their detection limits.

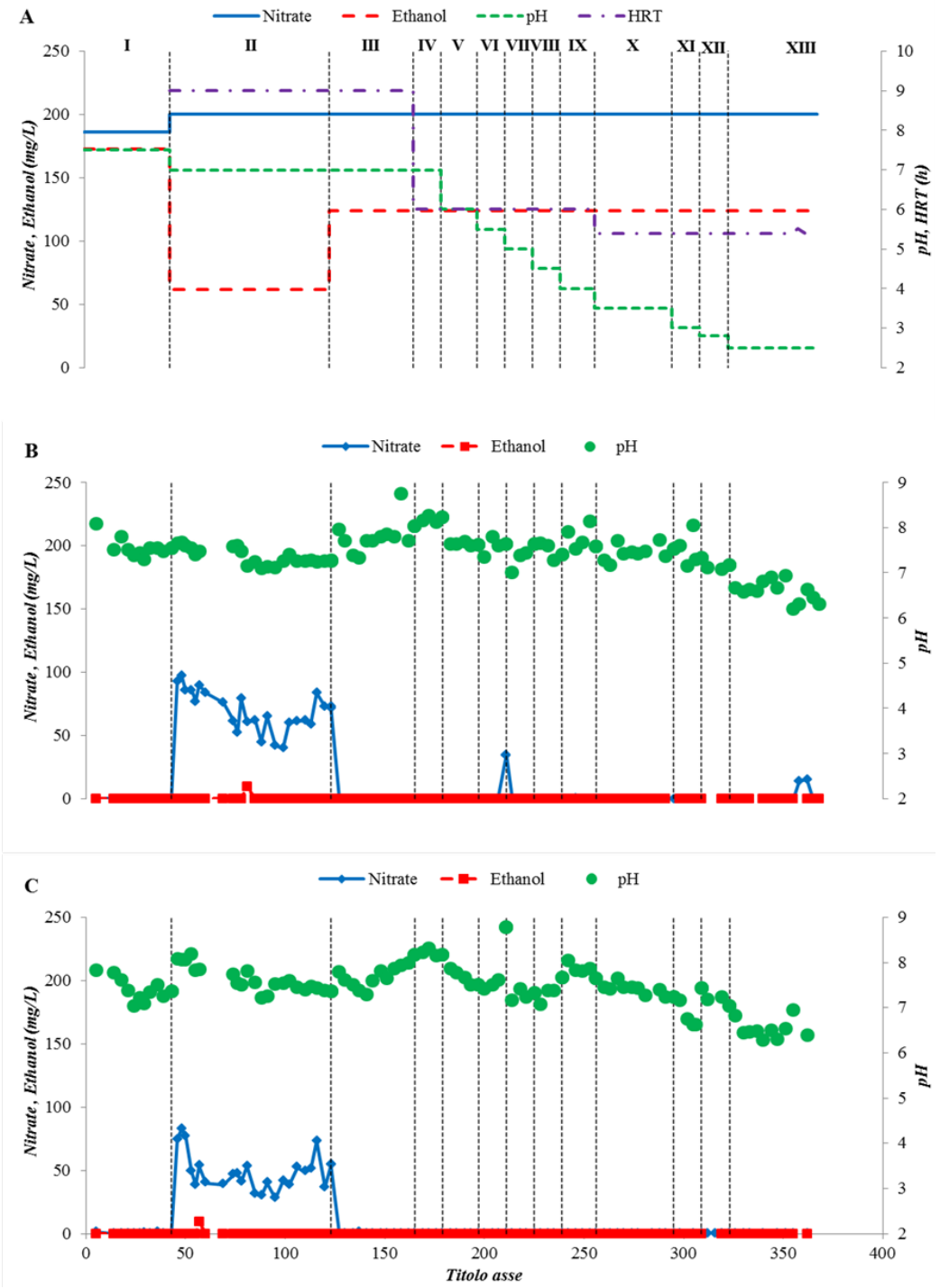

Figure 27 - The effects of pH and HRT on denitrification in fluidized-bed reactors at 7-8°C and 22°C

In period II, the FBRs were operated continuously at a 9h HRT and the ethanol/nitrate ratio was decreased from 1.25 to 0.42 (mol/mol). This decreased the denitrification efficiencies. FBR2 responded better to the feed and hydraulic condition changes The average nitrate concentration in the effluent raised to 69 and 49 mg/L at 7-8°C and 22°C, respectively, accompanied by nitrite accumulation in both FBRs (Figure 28). Although nitrate removal was incomplete, ethanol remained below the detection limit, giving evidence of the presence of different active ethanol-utilizing bacteria in the original seed.

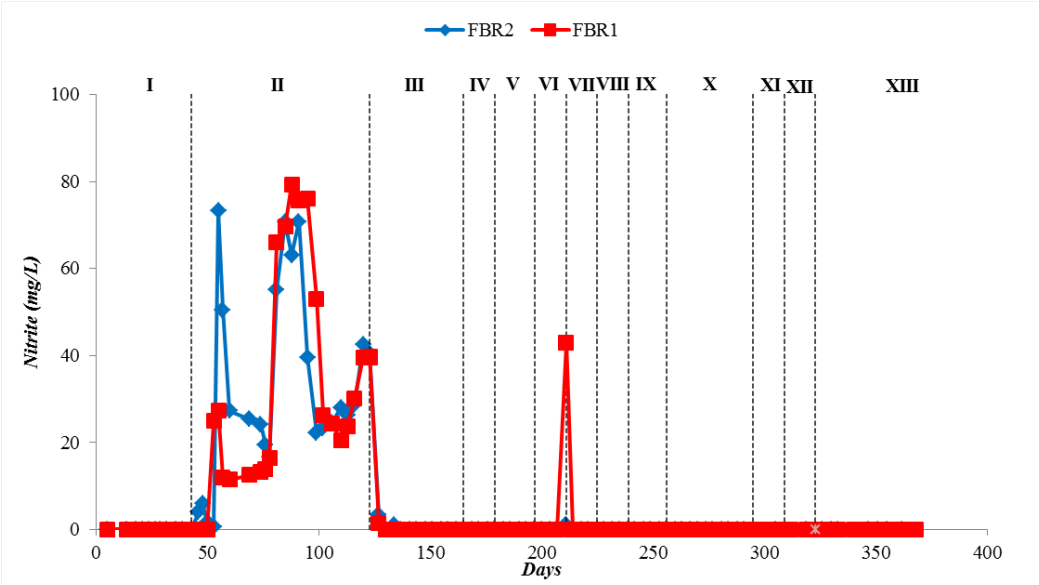

Figure 28 – Nitrite accumulation in FBR1 (7-8°C) and FBR2 (22°C)

From period III on, nitrate was removed after increasing the feed ethanol/nitrate ratio to 0.83. The oversupply of ethanol (two times in excess to the stoichiometric conditions reported in reaction 3) stimulated the activity of denitrifying bacteria. The following gradual feed pH and HRT decreases from 7. to 2.5 and from 9h to 5.4h, respectively, did not affect the performances.

Ethanol and nitrite remained below the detection limits and the production of alkalinity neutralized the acidic feed.

4.3.3 Microbial communities

DGGE analysis was performed two times to reveal changes in the microbial communities at different temperatures during the enrichment phase and operation of the FBRs (Figure 29). In the analyses twenty DGGE sequences were obtained. Dendrogram in Figure 30 shows the relationship between the bacterial sequences obtained by DGGE.

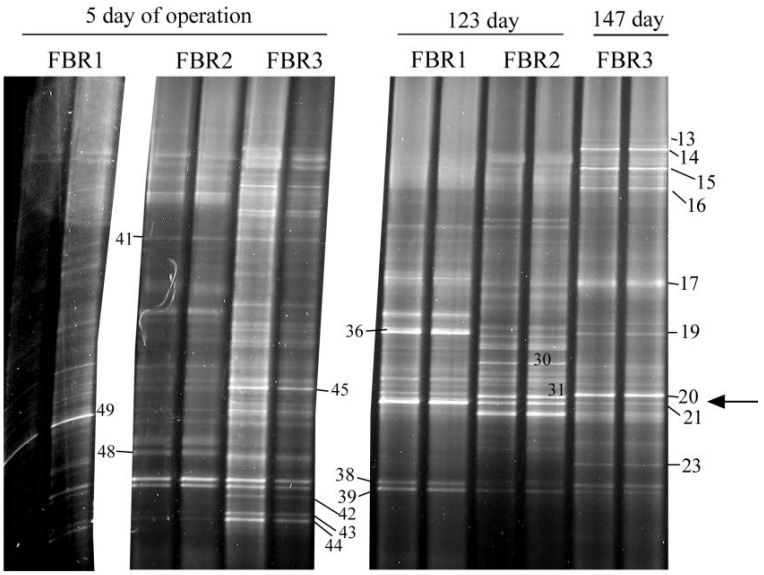

Figure 29 - DGGE profiles of microbial cultures enriched on ethanol in FBRs

Significant change in the FBR1 and FBR2 communities developed during the first 123 days of operation, as shown in bands 21, 30, 31 and 36 (black arrow in Figure 29). One denitrifying bacterium *Dechloromonas denitrificans* (band 21)

was present both at 7-8 and 22°C. The intensity of this band, especially in 7-8°C FBR, indicates that this bacterium was abundant and responsible for denitrification. Band 30 representing also *Dechloromonas denitrificans* was missing from FBR1 DGGE sequences during 123 days of operation. *Hydrogenophaga caeni* (band 31) was enriched to both reactors, more intensive at 22°C than 7-8°C. A strong band (band 36) was detected at 7-8°C but not in 22°C FBR and it could only be identified at the level of β-proteobacteria. During the operation of the FBRs from day 5 to day 123, bacteroidetes (sequence bands 13, 14, 15, 16) disappeared from FBR1 and FBR2. Nitrospirae bacteria (bands 38 and 39) were detected in both FBR1 and FBR2. These species were detected also at the first sampling point, after 5 days of operation.

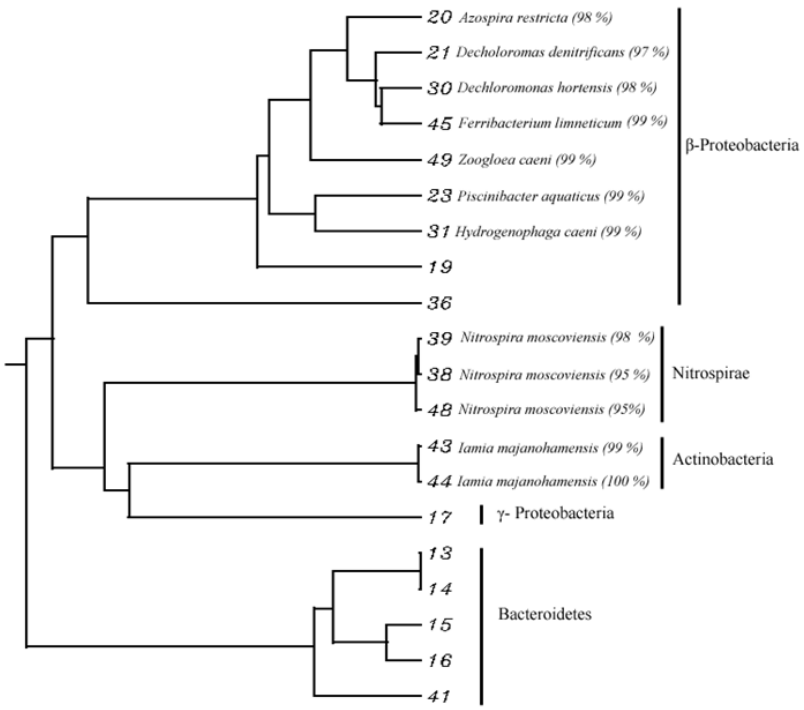

Figure 30 - Dendrogram showing the relationships between the bacterial sequences extracted from the DGGE gel

Chapter 5

General Discussion

5.1 METAL PRECIPITATION

5.1.1 Metal depletion kinetic parameters

Metal depletion kinetics and removal mechanisms in a sulfidogenic reactor highly depend on the sulfide concentration. The depletion rates of the metals when sulfide was added followed the same precipitation order according to the solubility product of metal sulfides (Sampaio et al., 2009). Sequential precipitation in a multi-metal system by the manipulation of sulfide concentration and pH has been described previously (Veeken et al., 2003a; Sahinkaya et al., 2009; Sampaio et al., 2009). Sampaio et al. (2009) achieved selective recovery of Cu over Zn due to the difference in solubility products between CuS and ZnS.

The different metal depletion rates from the plots of Zn, Cu, Pb and Cd removal for the different sulfide concentrations support the first-order kinetic model as also found by Mishra and Das (1992) and Lewis and Swartbooi (2006). In their works, the authors showed a better data fit with a standard deviation close to 1 for Zn depletion, in contrast with the current study. Due to the presence of macronutrients and a biofilm-coated polyethylene support, metal sulfide precipitation was interfered by competing precipitation reactions (Brezonik, 1994). CuS removal occurred very quickly confirming the results of Sahinkaya et al. (2009) who observed a Cu precipitation within 30-50 minutes in batch tests using biogenic sulfide at different metal to sulfide ratios.

5.1.2 Metal removal in absence of sulfide

This study also shows that metal sulfide precipitation is favored when sulfide concentration is maintained at quite high levels (40 mg/L in the present work).

The differences between the experiments with and without biofilm when sulfide was not externally added clearly show that Zn and Cd are partly adsorbed on the surface of biofilm. A comparison between bioprecipitation and biosortpion with SRB was carried out by Pagnanelli et al. (2010). The authors confirmed that Cd was mainly (77%) removed by a biosorption mechanism on the SRB cell wall surface. Another metal removal mechanism is the precipitation with components of the synthetic wastewater prepared for the batch experiments. XANES analysis confirms that Zn phosphate precipitation contributed to Zn removal, since the spectra of Zn precipitates in experiment S1 effectively fit the Zn phosphate spectra.

5.2 SULFATE-REDUCING IFB REACTORS

5.2.1 Sulfate reduction performances

Polypropylene was chosen as carrier material for the growth of a sulfate-reducing biofilm. After 35 days of IFBR operation, 83% and 74% of COD was removed in IFBR1 and IFBR2, respectively showing an appreciable enrichment of the biomass on the carrier. A similar start-up time was adopted in the study of Villa-Gòmez et al. (2011), who used lactate as carbon source to feed two IFBRs with two different COD/SO_4^{2-} ratios. Similarly to the present study, they obtained the highest COD removal efficiency (68%) in the reactor operated with the lowest COD/sulfate ratio ($COD/SO_4^{2-} = 1$) and the highest sulfate reduction efficiency (88%) in the reactor operated with the highest COD/sulfate ratio ($COD/SO_4^{2-} = 5$).

The start-up phase of both reactors was carried out using a stoichiometric COD/SO_4^{2-} ratio of 0.67 (reaction 1). Applying the same ratio of 0.67, Celis-

Garcia et al. (2007) observed that SRB could predominate over methanogens or other anaerobic microorganisms when excess lactate compared to propionate and butyrate was used in the organic mixture feed. The percentage of COD used for sulfate reduction was 80% and the COD removal and sulfate reduction efficiencies were 90% and 73%, respectively. In the present study, both reactors were seeded with a heterogeneous sludge taken from a classical anaerobic digester. Initially, SRB did not show to acclimate quickly to the system, resulting in sulfate removal percentages of 19% and 20% in IFBR1 and IFBR2, respectively. The COD percentage used for sulfate reduction was 35% in both reactors. This agrees with Sahinkaya and Gungor (2010) who obtained mean sulfate reduction percentages of 19% and 14% during the start-up of an up-flow fluidized-bed reactor and a down-flow fluidized-bed reactor, respectively.

A stoichiometric COD/SO_4^{2-} ratio has been demonstrated not to be the best to develop a biological sulfate-reducing process. In IFBR1, fed with a COD/SO_4^{2-} ratio of 0.67, the highest sulfate reduction efficiency was 35%, whereas a 97% of sulfate was reduced in IFBR2 on day 150 with a COD/SO_4^{2-} ratio of 4. This confirms the results of Sahinkaya and Gungor (2010), reporting that, in sulfidogenic reactors inoculated with a mixed anaerobic sludge, the added organic substrate was not only used for sulfate reduction but also for biomass growth, fermentation and methanogenesis. Therefore, it is more convenient to run a reactor aimed at sulfate reduction with higher COD/SO_4^{2-} ratios than the theoretical one. Also Velasco et al. (2008), operating a sulfidogenic ethanol-fed reactor, observed the lowest hydrogen sulfide production with the stoichiometric COD/SO_4^{2-} ratio, whereas the highest sulfide production of 470 mgS/L and the highest sulfate reduction efficiency of 94% were obtained with a COD/SO_4^{2-} ratio of 2.5. Similarly, Villa-Gòmez et al. (2011) obtained higher performances (i.e. 88% as sulfate reduction efficiency) in a IFBR operated with a COD/SO_4^{2-}

ratio of 5 than in a IFBR operated with a COD/SO_4^{2-} ratio of 1 (i.e. 68% as sulfate reduction efficiency).

IFBR2 was operated under different COD/SO_4^{2-} conditions and with sudden pH decreases in order to test the robustness of the system. Sulfate reduction was completely inhibited during periods II, IV and V when the influent pH was intentionally decreased from 5 to 3. On the contrary, Sahinkaya and Gungor (2010) and Kaksonen et al. (2003a) observed that sulfate reduction could also occur with influent pH values as low as 2 and 2.5, respectively, using lactate as electron donor. The reason for the failure of the process is the low fluidization degree adopted. Since the beginning of the experiment, it was noticed that the polypropylene beads were not adequate to guarantee a satisfactory biomass immobilization at fluidization degrees higher than 10%. It was necessary to keep the recycle rate low in order to avoid the biomass wash-out. Figure 31 shows the difference between the support colonization when higher (25%) and lower (10%) recirculation rate conditions were applied to the reactors, respectively. Lower fluidization degrees are recommendable in the start-up phase for enabling the biofilm formation, but it is advantageous to have higher fluidization degrees at increased loading rates or in presence of inhibitors (Garcia-Calderon et al., 1998b; Kaksonen et al., 2003a). When the pH 3-solution was fed to IFBR2, compounds such as lactate, acetate and sulfide were mainly present in their non-dissociated forms that are the most toxic ones (Kimura et al., 2006). Because of the low recycle rate, the system could not dilute their concentrations and thus the biomass activity was inhibited, resulting in a quick decrease of the effluent pH to 3.

Figure 31 - Support colonization at higher (A) and lower (B) fluidization degrees

5.2.2 Acetate production

Acetate accumulation in IFBR1 was one of the reasons of the lower efficiencies in terms of sulfate reduction. Very few SRB are able to oxidize lactate to carbon dioxide whereas most of them incompletely oxidize lactate to acetate. Acetate oxidation has been found to be the rate-limiting step in sulfate-reducing processes also fed with ethanol (Nagpal et al., 2000a; Kaksonen et al., 2003b; Gallegos-Garcia et al., 2008) and volatile fatty acids (Lens et al., 1998; Celis-Garcia et al., 2007). In IFBR1, lactate was always below the detection limit, indicating the conversion of lactate to acetate. Starting from day 49, acetate was detected in concentration of about 200 mg/L and from day 186 its average concentration increased to 250 mg/L. The lack of acetate-oxidizing sulfate reducers stopped the sulfate reduction as in Gallegos-Garcia et al. (2008).

Although higher acetate concentrations were detected in IFBR2 (up to 2322 mg/L), the sulfate reduction process showed not to be inhibited in Period III. Providing lactate in excess to sulfate, SRB could use lactate as electron donor even though it was converted partially to acetate. The more acetate accumulated in solution, the higher the sulfate reduction efficiencies were. As a confirmation, during periods II, IV and V sulfate reduction was almost totally absent and very little concentrations of acetate were detected in solution.

5.2.3 Effluent pH evolution

IFBR1 was not characterized by high performances and at most 61% of COD was used by SRB. However, the system was always able to neutralize the influent pH of 5. An important advantage of using lactate, compared to other electron donors such as ethanol, is that bicarbonate ions are produced even though lactate is only partially oxidized to acetate (Oyekola et al., 2009). In IFBR2, the influent acidity was neutralized only during periods III and VI as a consequence of the evolution of the sulfate-reducing process.

5.3 NITRATE-REMOVING APPLICATIONS

5.3.1 Denitrification performance

In FBRs, a molar C/N ratio of 2.5, using ethanol as carbon source, was shown to be very favorable for the growth of denitrifying bacteria from an activated sludge seed. As also found by Heylen et al. (2006), the activity of denitrifiers was quickly stimulated by the oversupply of ethanol both at 7-8°C and 22°C. Moreover, the batch FBR operation contributed to nitrate removal. During the

whole start-up phase, ethanol remained below the detection limit because of the activity of different ethanol-utilizing bacteria in the original seed.

The influence of two different temperature conditions on the denitrifying microbial cultures enriched on ethanol in FBRs was investigated. For all the duration of the experiment, the FBRs did not show different behaviors. The effect of low temperature on denitrification was studied by Zaitsev et al. (2008) who operated a denitrifying fixed-bed biofilm reactor at 5°C. Contrarily to this work, the authors found that denitrification was unstable (30-70% of nitrate removal) during the first six months of the experiment, most likely due to slow growth of methylotrophic denitrifying bacteria at5°C. On the other hand, Martin et al. (2009) reported the feasibility of removing 200 mg/L of nitrate in contaminated groundwater by injecting ethanol as microbial carbon source at a temperature of 6°C.

Ethanol seems to be a superior electron donor for biological denitrification as also demonstrated by dos Santos et al. (2004). The authors studied the effect of three different electron donors (methanol, ethanol and methane) on denitrification in batch tests finding out that ethanol was the most effective one. 90 mg/L of nitrate were completely reduced in 50 minutes using 38.3 mg/L of ethanol in 30°C-controlled batch reactors. In the present work, the ethanol/nitrate ratio resulted to be the most critical parameter to control. The excess of ethanol over nitrate continuously guaranteed the required carbon source for the bacteria to reduce nitrate in an effective way. When ethanol was fed to the system with an ethanol/nitrate ratio of 0.83 (mol/mol), 200 mg/L of nitrate were removed in FBR1 and FBR2 even with a feed pH of 2.5 and a 5.4 h HRT.

The effect of acidic pH on denitrification was also studied. Literature lacks studies focused on the effects of very low pH on the activity of denitrifying

bacteria. Denitrification at low pH was first carried out and monitored using batch assays. Bacteria showed to strongly adapt to acidic conditions as a nitrate removal percentage of 77% and an effluent pH of 6.1 were obtained, starting from a feed pH of 3.5. A feed pH value of 3.0 inhibited the process. Only 47% of nitrate removal was achieved and the pH was increased to 4.8. The pH effect was then studied in the FBRs by gradually decreasing the feed pH. FBRs showed to have the potential to remove nitrate and ethanol and recycle the alkalinity produced neutralizing the feed pH of 2.5.

5.3.2 Revealing of microbial communities

Microbial community was monitored during the reactors start-up at period I and period II by DGGE based on 16S rRNA gene. Microbial community changed during the 123 days. Because of the reactor conditions, selective pressure induced active bacterial strains to become abundant and dominant. This was clearly detected by DGGE as strong bands.

Denitrification was shown to occur at both operational temperatures. *Dechloromonas denitrificans* was detected both in FBR1 and FBR2, strongly indicating that this strain was mainly responsible for denitrification. Growth temperature reported for *Dechloromonas denitrificans* is wide from 5 to 36°C, explaining why it was grown both at 7-8°C and 22°C (Horn et al., 2005). *Hydrogenophaga caeni* was also found in both reactors and previously isolated from microbial communities of activated sludge (Shao et al., 2009). This strain is not reported to be able for denitrification but it is interesting to notice that this strain has been shown to reduce nitrate to nitrite (Shao et al., 2009). Second sampling for DGGE was performed at the end of the period II where

ethanol/nitrate ratio was decreased inducing nitrite accumulation and decrease in nitrate removal efficiencies.

Two bacterial strains *Nitrospira moscoviensis and Zoogloea caeni* found their niche in FBRs by utilizing the metabolic products of denitrification bacteria. *Nitrospira* belonging *N. moscoviensis* strain gain energy from oxidation of nitrite to nitrate using CO_2 as a carbon source. *Zoogloea caeni* previously isolated from activated sludge has shown to be capable for nitrate reduction to nitrogen gas but also nitrogen fixation (Chung et al., 2007).

Chapter 6

Conclusions

This study demonstrated that sulfate reduction and denitrification bioprocesses can be successfully applied to the treatment of acidic metal-, sulfate- and nitrate-contaminated mine waters. Metal sulfide precipitation can only be achieved when the sulfide concentration is maintained above the stoichiometric value. Under these sulfide conditions, Zn, Cu, Pb and Cd precipitated all within 9 hours. In this multi-metal system, Zn showed to be the metal with the highest solubility product and it resulted be removed by mechanisms other than metal sulfide precipitation. Biosorption, precipitation with macronutrients as phosphate and sorption onto its precipitates strongly contributed to Zn removal.

FBR and IFBR technologies were used for carrying out denitrification and sulfate reduction, respectively. In the two sulfidogenic IFBRs, low-density polypropylene pellets were used as carrier but they showed to be inadequate to attain a satisfactory immobilization of the biomass with fluidization degrees higher than 10%. The necessity to use a 10% fluidization degree led to the failure of the sulfate reduction when a feed pH of 3 was used. Operating the IFBRs with a COD/sulfate ratio of 4 and a feed pH of 5, a significant sulfate removal was obtained (higher than 95%). On the other hand, using a COD/SO_4^{2-} ratio of 0.67, acetate production and microbial competition limited the SRB activity to a 35% sulfate reduction efficiency.

Generally, in a AMD-treating system, low electron donor concentrations are sufficient to obtain an almost complete metal precipitation as sulfide is produced in enough quantities and metal sulfide solubility products are low. On the contrary, if the aim is to optimize sulfate reduction, higher COD/SO_4^{2} ratios promote SRB activity. Thereby, even if other fermentative reactions occur or acetate accumulates, enough electron donor is available for the sulfate-reducing activity.

Two classical FBRs were suitable for maintaining stable denitrification under low-pH conditions even at 7-8°C. The feed ethanol/nitrate ratio was an important control parameter. Adding ethanol in excess to nitrate, 200 mg/L of nitrate were reduced both in reactors and batch assays. Under ethanol in excess to nitrate conditions, the gradual feed pH and HRT decreases from 7 to 2.5 and from 9 to 5.4 h, respectively, did not affect denitrification.

DGGE analyses showed strong and several nitrate-reducing bacterial cultures enriched on ethanol colonizing the support of the FBRs. *Dechloromonas denitrificans* and *Hydrogenophaga caeni* were shown to indifferently grow up both at 7-8 and 22°C. *Zoogloea caeni* was definitely more enriched at 7-8°C. The electron flow for purposes different from denitrification occurred, also shown by the presence of other ethanol-utilizing microorganisms.

Chapter 7

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