

Nitrate removal and Fe(III) recovery through Fe(II)-driven denitrification with different microbial cultures

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Abstract

Ferrous iron mediated autotrophic denitrification is an innovative bioprocess for nitrate removal, simultaneously with iron oxidation in wastewaters. Chemoautotrophic denitrifiers convert nitrate to nitrogen gas and Fe(II) oxidation results in the production of ferric iron precipitates that can be subsequently removed and recovered. Both mixed enrichments and pure cultures isolated from various habitats have been reported to perform nitrate-dependent Fe(II) oxidation, especially at circumneutral pH. Under these conditions, Fe(II) is not stable and a chelating agent, commonly ethylenediaminetetraacetic acid (EDTA), needs to be employed in order to promote a higher Fe(II) solubilization. Different biogenic Fe(III) (hydro)oxide mineral phases can be formed, depending to many factors such as microorganisms, medium composition and incubation conditions.

The feasibility of maintaining Fe(II)-mediated autotrophic denitrification with a *Thiobacillus* mixed culture, an activated sludge inoculum and pure cultures of *Pseudogulbenkiania* strain 2002 and *T. denitrificans* under different pH and EDTA:Fe(II) conditions was initially investigated in batch bioassays. Lower EDTA: Fe(II) ratios resulted in higher nitrate removal efficiency and rates. After a longer acclimation to Fe(II) and stimulation with $S_2O_3^{2-}$, the *Thiobacillus* mixed culture resulted in the highest specific nitrate removal rate, equal to 10.5 mg/(g VSS·d).

Subsequently, the *Thiobacillus* mixed culture was seeded in two identical up-flow packed bed reactors (PBRs) in order to optimize the operating parameters such as the hydraulic retention time (HRT) and the nitrate loading rate and evaluate their influence on nitrate removal and Fe(II) oxidation during 153 d of operation. The HRT was shortened from 31 to 20 h. During the steady state the maximum nitrate volumetric loading rate (VLR) and volumetric removal rate (VRR) were 280 and 240 mg NO_3^- /(L·d) for PBR1 and 210 and 160 mg NO_3^- /(L·d) for PBR2, respectively. Results showed that the nitrate removal rate increased at increasing feed nitrate concentrations. At the steady state, nitrate removal and Fe(II) oxidation were $86\pm2\%$ and $95.5\pm2.5\%$, respectively, in PBR1, whereas $65.5\pm3.5\%$ and $70.0\pm2\%$, in PBR2. The lower initial feed NO_3^- concentration resulted in lower efficiencies in PBR2.

Moreover, the influence of heavy metals (Ni, Cu, Zn) was assessed in batch bioassays. The highest nitrate removal efficiency and rates were achieved with the *Thiobacillus*-dominated mixed culture, whereas *Pseudogulbenkiania* strain 2002 was the least effective. At initial 20.0 - 40.0 mg Me/L, Cu showed to be the most inhibitory metal for mixed cultures, resulting in an inhibition in the range 33-66%. A lower impact was observed when Zn was supplemented, leading to a 17-41% inhibition of nitrate removal. Ni showed the lowest inhibitory effect, with nitrate removal that was affected by 5-34% at Ni concentrations from 20.0 to 40.0 mg Ni/L. A higher sensitivity to metal toxicity was observed for the pure cultures.

Finally, the mineral characterization of the precipitates obtained in the experiments with Cu, Ni and Zn was investigated by different methods, i.e. scanning electron microscopy (SEM), Fourier transformation infrared spectroscopy (FTIR), Raman spectroscopy and X-ray fluorescence (XRF). All samples contained ferrihydrite. In abiotic controls, the chemical Fe(II) oxidation resulted in hematite formation and goethite or akaganeite. Precipitates of the experiments carried out with the *Thiobacillus*-dominated mixed culture were a mixture of hematite and akaganeite. When *T.denitrificans* was used, hematite and maghemite were observed. The use of pure culture of *Pseudogulbenkiania* strain 2002 resulted in hematite and maghemite formation and the activated sludge enrichment allowed the production of hematite and maghemite and/or magnetite. No difference in the mineralogy of the precipitates was observed with the addition of Cu, whereas the addition of Ni and Zn markedly stimulated the formation of maghemite. Heavy metals in the precipitates were identified by chemical analysis, indicating co-precipitation and/or absorption in Fe(III) (hydr)oxides.

Sommario

La denitrificazione autotrofa mediata da ferro ferroso è un processo innovativo per la rimozione dei nitrati, con la simultanea ossidazione del ferro, in acque reflue. I denitrificatori chemoautotrofi convertono il nitrato ad azoto gassoso e l'ossidazione del Fe(II) produce precipitati di ferro ferrico che possono essere successivamente rimossi e recuperati. Sia le colture arricchite miste che quelle pure, isolate da vari habitat, sono state indicate in grado di eseguire l'ossidazione del Fe(II) nitrato-dipendente, specialmente a pH vicino alla neutralità. In queste condizioni, il Fe(II) non è stabile e richiede l'utilizzo di un agente chelante, come l'acido etilendiamminotetraacetico (EDTA), affinché si possa raggiungere una più elevata solubizzazione del Fe(II). Possono formarsi diverse fasi minerali biogeniche di (idr-)ossido di Fe(III), a seconda di molti fattori come microorganismi, composizione del mezzo e condizioni di incubazione.

In questo studio è stata investigata la fattibilità di mantenere la denitrificazione autotrofa mediata da Fe(II) utilizzando una coltura mista di *Thiobacillus*, fango attivo come inoculo e una coltura pura del ceppo 2002 di *Pseudogulbenkiania* e *T. denitrificans*, in batch a differenti condizioni di pH e rapporto EDTA:Fe(II).

Successivamente, la coltura mista di *Thiobacillus* è stata inoculata in due reattori a letto impaccato (PBR) così da ottimizzare parametri operativi come il tempo di ritenzione idraulico (HRT) e il carico di nitrato, ed in questo modo valutare la loro influenza sulla rimozione di nitrato e l'ossidazione del Fe(II) durante i 153 giorni di operatività. L'HRT è stato accorciato da 31 a 20 ore. Durante la fase stazionaria, il massimo carico organico volumetrico (VLR) e il massimo tasso di rimozione volumetrico (VRR) di nitrato, erano 280 e 240 mg NO₃⁻/(L·d) per il PBR1 e 210 e 160 mg NO₃⁻/(L·d) per il PBR2, rispettivamente. I risultati hanno mostrato un tasso di rimozione del nitrato crescente all'aumentare della concentrazione di nitrato in entrata. Allo stato stazionario, la rimozione dei nitrati e l'ossidazione di Fe (II) erano 86 ± 2% e 95,5 ± 2,5%, rispettivamente, nel PBR1, mentre 65,5 ± 3,5% e 70,0 ± 2%, nel PBR2. La minore concentrazione iniziale di NO₃⁻ ha portato a minori efficienze nel PBR2.

Inoltre, è stata valutata l'influenza di metalli pesanti (Ni, Cu e Zn) attraverso uno studio in condizioni batch. La più alta efficienza e il maggiore tasso di rimozione del nitrato sono stati ottenuti utilizzando la coltura mista dominata da *Thiobacillus*, mentre il ceppo 2002 di *Pseudogulbenkiania* è stato il meno efficace. A valori iniziali di 20.0-40.0 mg Me/L, Cu ha dimostrato di essere il metallo più inibitore per le colture miste, con conseguente inibizione nell'ordine di 33-66%. Un impatto inferiore è stato osservato con Zn, che ha portato ad un'inibizione del 17-41% della rimozione di nitrati. Ni ha mostrato il più basso effetto inibitorio, con la rimozione del nitrato che è stata influenzata del 5-34% alle concentrazioni di Ni da 20.0 a 40.0 mg Ni/L. Le colture pure hanno mostrato una maggiore sensibilità alla tossicità del metallo.

Infine, la caratterizzazione minerale dei precipitati ottenuti negli esperimenti con Cu, Ni e Zn è stata analizzata mediante diversi metodi: microscopia elettronica a scansione (SEM), spettroscopia infrarossa a trasformazione di Fourier (FTIR), spettroscopia Raman e fluorescenza a raggi X (XRF). Tutti i campioni contenevano feridrite. Nei controlli abiotici l'ossidazione chimica di Fe(II) ha provocato la formazione di ematite e goethite o akaganeite. I precipitati degli esperimenti condotti con la coltura mista dominata dal *Thiobacillus* erano una miscela di ematite e akaganeite. Quando è stato utilizzato *T.denitrificans*, sono state osservate ematite e maghemite. L'uso della coltura pura del ceppo 2002 di *Pseudogulbenkiania* ha portato alla formazione di ematite e maghemite e l'arricchimento dei fanghi attivi ha permesso la produzione di ematite e maghemite e/o magnetite. Non è stata osservata alcuna differenza nella mineralogia dei precipitati aggiungendo Cu, mentre l'aggiunta di Ni e Zn ha notevolmente stimolato la formazione di maghemite. I metalli pesanti nei precipitati sono stati identificati mediante analisi chimiche, indicando co-precipitazione e/o assorbimento in (idr-)ossidi di Fe (III).

Résumé

La dénitrification autotrophe utilisant le fer Ferreux est un bioprocédé innovant pour l'élimination des nitrates tout en permettant en même temps l'oxydation du fer dans les eaux usées. Les bactéries dénitrifiantes chimio-autotrophes convertissent les nitrates en azote gazeux et l'oxydation du Fe(II) conduit à la production de précipités de fer ferrique qui peuvent ensuite être éliminés et récupérés.

La possibilité de maintenir une dénitrification autotrophe avec le fer ferreux en utilisant une culture mixte de *Thiobacillus*, un inoculum de boue activée et des cultures pures de *Pseudogulbenkiania* 2002 et de *T. denitrificans* dans différentes conditions de pH et d'EDTA:Fe(II) a été initialement étudiée dans des essais en mode batch. Des ratios plus faibles d'EDTA:Fe(II) se traduisent par une efficacité et des taux d'élimination des nitrates plus élevés. La culture mixte de *Thiobacillus*, qui présente le taux d'élimination de nitrate le plus élevé, est égal à 1,18 mM·(g VSS·d)⁻¹.

Par la suite, la culture mixte de *Thiobacillus* a étéensemencée dans deux réacteurs à lit fixe à flux ascendant identiques. Les deux réacteurs (réacteur 1 et 2) ont reçu respectivement 120 et 60 mg L⁻¹ de nitrate et une alimentation différente de Fe (II) afin de respecter un rapport molaire Fe(II):NO₃⁻ de 5:1. L'EDTA a été supplémenté à un rapport molaire EDTA:Fe(II) de 0,5:1. Le pH, le temps de séjour hydraulique (TSH) et la température étaient de 6,5-7,0, 31 h et 22 ± 2 ° C. Dans le réacteur 1, le TSH a été raccourci de 31 à 24 h et la concentration de NO₃⁻ a été maintenue stable à 250 mg L⁻¹. Inversement, le réacteur 2 a été mis en fonctionnement avec un TSH décroissant et une concentration entrante de NO₃⁻ permettant de maintenir un taux de charge de NO₃⁻ stable. Après environ 80 jours d'incubation, l'élimination des nitrates était de 88% dans le réacteur 1 pour un TSH de 31 h. L'élimination de nitrates la plus élevée obtenue dans le réacteur 2 était de 80%. Une diminution du TSH de 31 à 24 h n'a pas affecté l'élimination des nitrates dans le réacteur 1, alors que dans le réacteur 2 l'élimination du nitrate a diminué à 64%.

De plus, l'influence des métaux lourds (Ni, Cu, Zn) sur la dénitrification autotrophe utilisant du fer ferreux a été évaluée dans des essais biologiques discontinus, en utilisant les mêmes cultures microbiennes. L'efficacité et les taux d'élimination des nitrates les plus élevés ont été obtenus avec la culture mixte dominante de *Thiobacillus*, alors que la souche *Pseudogulbenkiania* de 2002 était la moins efficace. Cu s'est avéré être le métal le plus inhibiteur pour les cultures mixtes. Un impact plus faible a été observé lorsque le Zn a été ajouté. Le Ni présentait l'effet inhibiteur le plus faible. Une sensibilité plus élevée à la toxicité des métaux a été observée pour les cultures pures.

Enfin, la caractérisation minérale des précipités obtenus pour les expériences avec du Cu, Ni et Zn a été étudiée. Pour les témoins abiotiques, l'oxydation chimique du Fe (II) a entraîné la formation d'hématite. Un mélange de différents (hydro)oxydes de Fe(III) a été observé pour toutes les cultures microbiennes, et en particulier : i) un mélange d'hématite, d'akaganéite et / ou de ferrihydrite a été observé dans les précipités des expériences réalisées avec la culture mixte dominée par la présence de *Thiobacillus*; ii) également l'hématite, l'akaganéite et / ou de la ferrihydrite, la maghémite a été identifiée lorsque la culture pure de *T. denitrificans* a été utilisée; iii) l'utilisation de la culture pure de la souche *Pseudogulbenkiania* 2002 a entraîné la formation d'hématite et de maghémite; enfin, l'enrichissement en boues activées a permis la production d'hématite et de magnétite en plus de la maghémite. Aucune différence concernant la minéralogie des précipités n'a été observée avec l'addition de Cu, alors que l'addition de Ni et de Zn a probablement stimulé la formation de maghémite. Une caractérisation minérale supplémentaire est cependant nécessaire.

Samenvatting

Ferro-ijzer gemedieerde autotrofe denitrificatie is een innovatieve bioproces voor nitraatverwijdering, gelijktijdig met ijzeroxidatie in afvalwater. Chemoautotrofe denitrificeerders zetten nitraat om naar stikstofgas en Fe(II) oxidatie resulteert in de productie van ferro-ijzer precipitaten die vervolgens kunnen worden verwijderd en teruggewonnen. Van zowel gemengde verrijkingen als zuivere culturen geïsoleerd uit verschillende habitats is gemeld dat ze nitraatafhankelijke Fe(II) oxidatie uitvoeren, vooral bij circumneutrale pH. Onder deze omstandigheden is Fe(II) niet stabiel en moet een cheleringsmiddel, gewoonlijk ethyleendiaminetetraazijnzuur (EDTA), worden gebruikt om een hogere Fe(II) -solubilisatie te bevorderen. Verschillende biogene Fe(III) (hydro) oxide minerale fasen kunnen worden gevormd, afhankelijk van vele factoren zoals micro-organismen, gemiddelde samenstelling en incubatie-omstandigheden.

De haalbaarheid van het onderhouden van Fe(II) -gemedieerde autotrofe denitrificatie met een *Thiobacillus* gemengde kweek, een actief slib inoculum en zuivere kweken van *Pseudogulbenkiania* stam 2002 en *T. denitrificans* onder verschillende pH en EDTA:Fe(II) omstandigheden werd aanvankelijk onderzocht in batch bioassays. Lagere EDTA:Fe (II) -verhoudingen resulteerden in een hogere efficiëntie en snelheden van nitraatverwijdering. Na een langere acclimatisering van Fe (II) en stimulatie met $S_2O_3^{2-}$, resulteerde de gemengde cultuur van *Thiobacillus* in de hoogste specifieke nitraatverwijderingssnelheid, gelijk aan $10.5 \text{ mg/(g VSS} \cdot \text{d)}^{-1}$.

Vervolgens werd de gemengde kweek van *Thiobacillus* geënt in twee identieke up-flow gepakte bedreactoren (PBR's) om de bedrijfsparameters zoals de hydraulische retentietijd (HRT) en de nitraatbeladingssnelheid te optimaliseren en hun invloed op nitraatverwijdering en Fe(II) te evalueren oxidatie gedurende 153 d bewerking. De HRT werd ingekort van 31 tot 20 uur. Tijdens de stationaire toestand waren de maximale nitraat-volumetrische belastingssnelheid (VLR) en volumetrische verwijderingssnelheid (VRR) 280 en 240 $\text{mg NO}_3^-/(\text{L} \cdot \text{d})$ voor PBR1 en 210 en 160 $\text{mg NO}_3^-/(\text{L} \cdot \text{d})$ voor PBR2, respectievelijk. De resultaten toonden aan dat de nitraatverwijderingssnelheid toenam bij toenemende nitraatconcentraties. Bij de steady-state waren nitraatverwijdering en Fe(II) -oxidatie respectievelijk $86 \pm 2\%$ en $95.5 \pm 2.5\%$, in PBR1, terwijl $65.5 \pm 3.5\%$ en $70.0 \pm 2\%$, in PBR2. De lagere initiële NO_3^- concentratie aan voer resulteerde in lagere efficiënties in PBR2.

Bovendien werd de invloed van zware metalen (Ni, Cu, Zn) beoordeeld in batch-bioassays. De hoogste nitraatverwijderingsefficiëntie en -snelheden werden bereikt met de door *Thiobacillus* gedomineerde gemengde kweek, terwijl *Pseudogulbenkiania* stam 2002 het minst effectief was. Aanvankelijk 20.0 - 40.0 mg Me/L , bleek Cu het meest remmende metaal te zijn voor gemengde kweken, resulterend in een remming in het bereik van 33-66%. Een lagere impact werd waargenomen wanneer Zn werd aangevuld, wat leidde tot een 17-41% remming van de verwijdering van nitraat. Ni vertoonde het laagste remmende effect, waarbij nitraatverwijdering werd aangetast door 5-34% bij Ni-concentraties van 20.0 tot 40.0 mg Ni/L . Een hogere gevoeligheid voor metaaltoxiciteit werd waargenomen voor de zuivere culturen.

Ten slotte werd de minerale karakterisering van de precipitaten verkregen in de experimenten met Cu, Ni en Zn onderzocht met verschillende methoden, dat wil zeggen scanning elektronenmicroscopie (SEM), Fourier transformatie infrarood spectroscopie (FTIR), Raman spectroscopie en X-stralen fluorescentie (XRF). Alle monsters bevatten ferrihydriet. Bij abiotische controles resulteerde de chemische Fe(II) -oxidatie in hematietvorming en goethiet of akaganeiet. Precipitaten van de experimenten uitgevoerd met de door *Thiobacillus* gedomineerde gemengde cultuur waren een mengsel van hematiet en akaganeiet. Wanneer *T. denitrificans* werd gebruikt, werden hematiet en maghemiet waargenomen. Het gebruik van zuivere kweek van *Pseudogulbenkiania* stam 2002 resulteerde in hematiet en maghemietvorming en de geactiveerde slikverrijking maakte de productie van hematiet en maghemiet en / of magnetiet mogelijk. Er werd geen verschil in de mineralogie van de precipitaten waargenomen met de toevoeging van Cu, terwijl de toevoeging van Ni en Zn de vorming van maghemiet duidelijk stimuleerde. Zware metalen in de precipitaten werden geïdentificeerd door chemische analyse, hetgeen co-precipitatie en/of absorptie in Fe (III) (hydro) oxiden aangeeft.

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CHAPTER 1

General introduction and Thesis Outline

1.1 Background and problem statement

Clean water is essential for human health and environment, therefore water quality is one of the most important concerns of European environmental policy. One of the first European Union (EU) legislation was the 1991 Nitrate Directive aimed to control pollution of water resources. Nitrogen is a vital nutrient for organisms, but, however, is harmful to people and nature ecosystems at high concentrations (European Union, 2000). Nitrate is one of the most common pollutants in water resources worldwide (Park and Yoo, 2009; Zhang *et al.*, 2015). The 2000 EU Directive on nitrate pollution reported that nitrate concentrations must not exceed the trigger value of 50 mg/L in drinking water (European Union, 2000). The main causes of nitrate contamination is the agricultural fertilizers and the uncontrolled discharge of industrial and domestic wastewaters (Viers *et al.*, 2012; Qambrani *et al.*, 2013; Zhang *et al.*, 2015).

The high costs of physicochemical methods for nitrate removal from wastewaters such as ion exchange, reverse osmosis, adsorption, and electrodialysis limit their applications (Shrimali *et al.*, 2001). Biological denitrification is a cost-effective technology for removal of nitrate from wastewaters, with high efficiencies and low energy consumption (Zhang *et al.*, 2015; Zhou *et al.*, 2016). Heterotrophic denitrification is the most used bioprocess for nitrate removal (Ashok and Hait, 2015). However, heterotrophic metabolism requires carbon source and many wastewaters contain no or too low carbon, while the addition of external organics can result in organic residues (Zhang *et al.*, 2015). Nitrate can often be found in mining environments due to the extensive use of nitrate-containing explosives such as ‘ammonium nitrate fuel oil’ (ANFO) (Zaitsev *et al.*, 2008). Mining wastewaters that are generated contain nitrate with several metals species, including iron (Papirio *et al.*, 2014).

The crucial dependency on raw materials such as metals and its supply risk is a growing concern for EU in the recent years. EU has set up a long-term strategy to reduce landfilling and increase recycling and recovery of these materials from “urban mines” and industrial waste to support the transition towards a circular economy (European Union, 2017). Nowadays, the need of iron is significantly higher, due to the growth of human population. The natural high-grade ores are rapidly decreasing (Yan and Wang, 2014), therefore the exploration of secondary sources of iron is crucial.

Fe(II)-mediated autotrophic denitrification is an alternative biotechnology to simultaneously remove nitrate and recover iron through the formation of Fe(III) precipitates.

The first microorganisms capable of maintaining biological nitrate-dependent Fe(II) oxidation were discovered only 20 years ago (Straub et al., 1996). The use of denitrifying Fe(II) oxidizers results in the reduction of nitrate to nitrogen gas and the bioprecipitation/biorecovery of Fe(III) (hydr)oxides, with the possible co-precipitation or adsorption of other metals (Hohmann et al., 2009; Ahoranta et al., 2016). Microorganisms capable of the process are neutrophilic, therefore ethylenediaminetetraacetic acid (EDTA) is commonly employed as chelating agent in order to promote a higher Fe(II) solubilization, as Fe(II) is not stable at circumneutral pH (Kiskira et al., 2017).

So far, nitrate dependent Fe(II) oxidation has been reported in both mixed enrichments and pure cultures isolated from various habitats, however most of the studies have been focused in the microbiology rather than developing the process as a biotechnology for nitrate removal and iron recovery. Further research is therefore needed in order to evaluate the Fe(II)-mediated autotrophic denitrification in continuous-flow applications.

1.2 Objectives and scope of the study

This research investigated the Fe(II) mediated autotrophic denitrification in order to achieve nitrate removal with an emphasis on the bioprecipitation/biorecovery of iron and other heavy metals by different microbial cultures. The specific objectives of this research are:

1. Investigation of the feasibility of maintaining denitrification with different EDTA:Fe(II) ratios, feed pH and microbial cultures within batch bioassays.
2. Investigation of the effect of other metals (Ni, Cu, Zn) on the process in batch bioassays.
3. Mineral characterization of the Fe(III)(hydr)oxides during Fe(II)-driven denitrification.
4. Evaluation of the process in packed bed reactors (PBRs) in order to evaluate the effect of different operating parameters (e.g. hydraulic retention time, feed nitrate concentration and nitrate loading rate) on the performance of Fe(II)-driven denitrification.

1.3 Thesis outline

This PhD thesis is divided into seven chapters. The first chapter (Chapter 1) provides a brief overview of this research and the thesis as depicted in Figure 1.1.

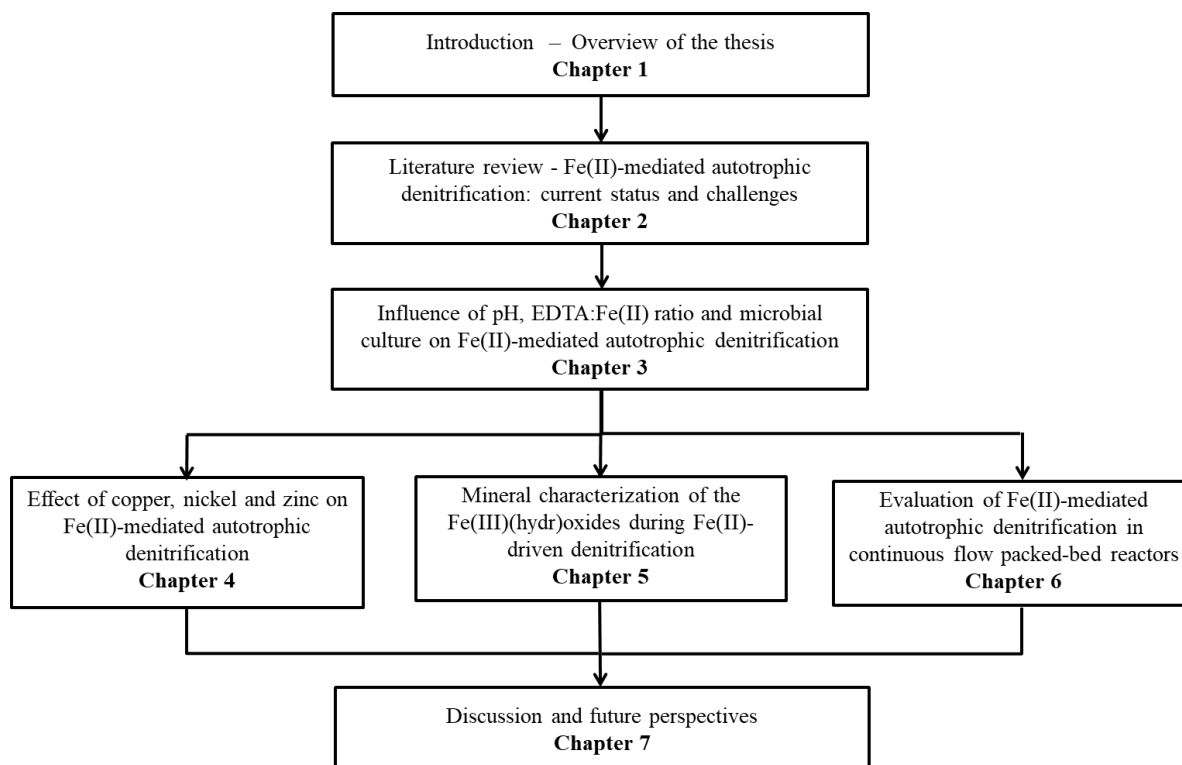


Figure 1.1: Overview of the PhD thesis structure

Chapter 2 reports a critical literature review about current knowledge on Fe(II)-mediated autotrophic denitrification, the characteristics of the Fe(II)-oxidizing denitrifiers, the operating conditions adopted in the existing studies, the characteristics of the produced Fe(III) hydr(oxides) and the influence of other metals on the process.

Chapter 3 describes the experimental results obtained investigating the efficiency of Fe(II)-mediated autotrophic denitrification in terms of Fe(II) oxidation and nitrate removal with different microbial cultures in batch bioassays, the effects of decreasing pH on the process and the investigation of the optimal EDTA/Fe(II) ratio.

Chapter 4 discusses the results obtained investigating the efficiency and rates of nitrate removal during Fe(II)-mediated autotrophic denitrification in the presence of Ni, Cu and Zn in batch bioassays. Moreover, the investigation of how the different microbial cultures, tolerated the effect of Ni, Cu and Zn was assessed.

Chapter 5 presents the mineral characterization of the Fe(III) (hydr)oxides by different methods, i.e. X-ray fluorescence (XRF), Raman spectroscopy, scanning electron microscopy equipped with energy dispersive X-Ray Analyser (SEM-EDX) and Fourier transformation infrared spectroscopy (FTIR).

In chapter 6, Fe(II) mediated autotrophic denitrification was studied in continuous-flow experiments using two identical up-flow packed bed reactors, in order to optimize the operating parameters (e.g. HRT, feed nitrate concentration and nitrate loading rate) and evaluate their effects on the process.

Chapter 7 highlights the major findings and the implications of the research and provides perspectives and recommendations for future works.

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CHAPTER 2

Fe(II)-mediated autotrophic denitrification: A new bioprocess for iron bioprecipitation/biorecovery and simultaneous treatment of nitrate-containing wastewaters

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2.1 Introduction

Iron (Fe) is the most abundant element (on weight basis) on the planet, in both lithosphere and biosphere (Johnson et al., 2012). From ancient times, Fe has played an important role in the society and is associated with industrial development and improvement of living standards. Fe is a soft metal with good ductility and malleability and has a substantial strength to ductility ratio (Yan and Wang, 2014). The applications of iron in the daily life are many and Fe processing for industrial purposes accounts for 95% of worldwide metal production (Ilbert and Bonnefoy, 2013).

However, iron is not only economically important but is also an essential micronutrient for most organisms, including humans. Fe is a component in many cellular compounds and is involved in numerous physiological functions. Iron is incorporated as a cofactor in many metalloproteins involved in vital metabolic pathways, including oxygen transport, DNA synthesis and electron transport (Kendall et al., 2012; Ilbert and Bonnefoy, 2013). The iron deficiency often leads to anemia that can cause several motor and mental impairments in children, low work productivity in adults and poor pregnancy outcome (Horton and Ross, 2003).

Iron production is primary or secondary. Primary production consists of iron extraction from natural ores, rocks and minerals. Worldwide both iron and steel industries are mostly reliant on Fe-based ores as primary sources of iron. In nature, iron is not usually found as free metal but in association with or covered by natural organic matter or in particles associated with phosphate (PO_4^{3-}) and arsenate (AsO_4^{3-}) (Kappler and Straub, 2005). Hematite (Fe_2O_3) and magnetite (Fe_3O_4) are the main iron-containing ores. Other common iron minerals are limonite ($\text{Fe}_2\text{O}_3 \cdot \text{H}_2\text{O}$), siderite (FeCO_3), pyrite (FeS_2) and ilmenite ($\text{FeO} \cdot \text{TiO}_2$). Furthermore, Fe can be found in minerals that are mixed or co-precipitated with other minerals (e.g., clays) (Kappler and Straub, 2005). Up to date, the major world reserves of iron ores and the main iron producers are in Australia, Brazil, Russia, China, India and Canada. Other important producers are Ukraine, Sweden, United States, Iran, Kazakhstan and South Africa. Brazil and Australia export more than half of the iron extracted worldwide (Statista, 2016).

Nowadays, due to the growth of human population, the need of iron for steel production is significantly higher. The natural high-grade ores are rapidly decreasing inducing the exploration of secondary sources of iron. Europe, United States, Japan and other

developed countries are adopting technologies for recycling scraps to be used for steel production (Yan and Wang, 2014). Industrial wastewaters, generated from activities such as metal plating, metal picking, mining, fertilizer industries, tanneries, batteries, electronic and electroplating, paper mills and pesticides, can act as artificial ores, as they can contain iron concentrations as high as 100 g/L (Gonzalez-Munoz et al., 2006; Agrawal et al., 2009; Fu and Wang, 2011).

Mining activity is the principal producer of acidic and heavy metal contaminated wastewaters. Rainwater passing through abandoned mines, waste materials (spoils) and reactive mineral wastes (tailings) results in acid mine drainage (AMD) caused by the oxidation of sulfide minerals (e.g. FeS_2) that release H^+ ions, metals and sulfate into water (Johnson, 2003; Johnson and Hallberg, 2005). The most common metal present in mining waters and AMD is iron, with concentrations generally higher than 500 mg/L, and in some cases up to 35 g/L (Johnson, 2003). Depending on the characteristics of the ores, various other metals and metalloids can be present, such as Al, Mn, As, Ba, Cd, Cu, Mo, Ni, Pb, Se, Co, Bi, Ga, In, Hg, Re, Sb, Sn, Te and Zn (Banks et al., 1997; Johnson, 2003) that can also affect iron precipitation and speciation.

In general, the conventional methods for iron removal and recovery are physicochemical and electrochemical (Fig. 2.1). Physicochemical methods include chemical precipitation, oxidation and filtration, coagulation/flocculation, flotation, ion exchange, adsorption and membrane technology such as nanofiltration and reverse osmosis (Gonzalez-Munoz et al., 2006; Dobson and Burgess, 2007; Polat and Erdogan, 2007; Akpor and Muchie, 2010; Barakat, 2011; Balaji et al., 2014). The electrochemical methods are electroextraction, electrodialysis, membrane electrolysis and electrochemical precipitation (Smara et al., 2005; Korzenowski et al., 2008; Ali et al., 2013; Tong et al., 2014). Despite the high removal/recovery efficiency, physicochemical and electrochemical methods are very expensive and not preferable, especially when metal concentrations are low (Schiewer and Volesky, 2000). Moreover, the high maintenance requirements and the instability of large volumes of sludge results in significant environmental concerns and a loss of valuable metals (Hoque and Philip, 2011; Jadhav and Hocheng, 2012)

Nowadays, the industrial activity has to deal with more and more stringent effluent discharge regulations that usually result in high operating costs making the entire process unprofitable. Therefore, it is of major importance to investigate innovative technologies for recycling and recovery of iron from secondary sources such as AMD and industrial

wastewaters (Papirio et al., 2013). Besides the conventional physicochemical methods for iron removal and recovery, biotechnologies are more widely used nowadays for their low cost and energy requirement while giving recovery yields higher than 90% (Hoque and Philip, 2011; Jadhav and Hocheng, 2012). Furthermore, biorecovery is a self-sustaining technology since the microbial population increases when the contaminant is present and decreases when the contaminant is limited (Akpore and Muchie, 2010).

Nitrate-dependent Fe(II) oxidation is a promising biotechnology to be employed for iron recovery when nitrogen and metal contamination coexist (Papirio et al., 2014a; Zou et al., 2015). Chemoautotrophic denitrifiers are capable of coupling Fe(II) oxidation to denitrification resulting in the reduction of nitrate to nitrogen gas and the production of Fe(III) precipitates that can be subsequently removed and recovered. This review article contains the current knowledge about Fe(II)-mediated autotrophic denitrification. Initially, a short summary of iron biogeochemistry and the environmental concerns of Fe(II) and nitrate are given. The attention is then focused on the characteristics of the Fe(II)-oxidizing denitrifiers, the operating conditions adopted in the existing studies, the characteristics of the produced Fe(III) hydr(oxides) and the influence of other metals on the process. Finally, the use of pyrite as an alternative electron donor is discussed.

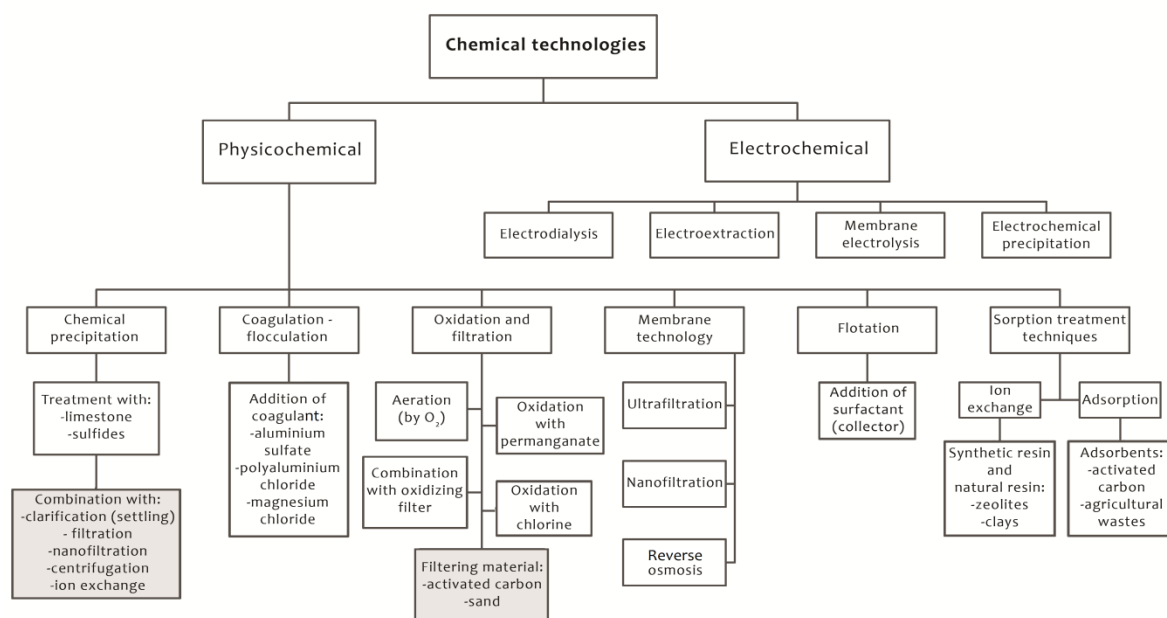


Figure 2.1: Chemical technologies for iron removal and recovery from wastewater.

2.2 Biochemistry of iron

Iron can exist in a range of oxidation states from -2 to $+6$, with Fe(II) and Fe(III) species being the most common ones (Ilbert and Bonnefoy, 2013). Fe oxidation state depends on many factors, such as pH, redox potential (Eh), temperature, dissolved oxygen (DO) and the presence or absence of other dissolved constituents such as complexing agents (Bird et al., 2011; Johnson et al., 2012; Kendall et al., 2012; Ilbert and Bonnefoy, 2013). Chemical and biological processes such as redox transformations, dissolution, precipitation, mobilization and redistribution rule iron biogeochemistry and occur in both terrestrial and aquatic environments (Kappler and Straub, 2005).

Chemical Fe(II) oxidation and Fe(III) precipitates formation are promoted at pH higher than 3 (Bird et al., 2011). At increasing pH and DO, Fe(II) oxidation rate increases (Kendall et al., 2012). Chemical Fe(II) oxidation can also occur under anaerobic conditions in presence of Mn(IV), NO_3^- , NO_2^- , N_2O (Kappler and Straub, 2005). Iron cycle is also influenced by microorganisms that generate energy for growth by the oxidation or reduction of iron (Bird et al., 2011). Microbial Fe(II) oxidation can occur in aerobic and anaerobic environments, while Fe(III) reduction is only common in anaerobic habitats (Kappler and Straub, 2005).

Fe(II) is more abundant in anaerobic environments and Fe(III) in aerobic, due to the rapid chemical oxidation of Fe(II) to Fe(III) by molecular oxygen (Johnson et al., 2012). Despite its geologic abundance, iron is often a growth limiting factor at circumneutral pH due to the formation of iron oxides, which are highly insoluble and, thus, not readily available for uptake by the most of the microorganisms (Johnson et al., 2012). In order to maintain Fe in solution, chelators (e.g. siderophores) can be used to form Fe complexes leading to higher soluble Fe concentrations, even at circumneutral pH (Bird et al., 2011).

The understanding of the iron cycle in aquatic environments is crucial as it influences the mobility, speciation and fate of organic and inorganic pollutants, including toxic trace metals and metalloids (Plach et al., 2014). The microbial iron cycle can be used in a variety of applications, such as the bioleaching of ores (Hedrich et al., 2011) and the bioremediation of mining waters contaminated with soluble Fe(II) (Johnson et al., 2012). The redox transformations of iron and precipitation and dissolution of iron minerals are the result of chemical and microbial processes (Fig. 2.2).

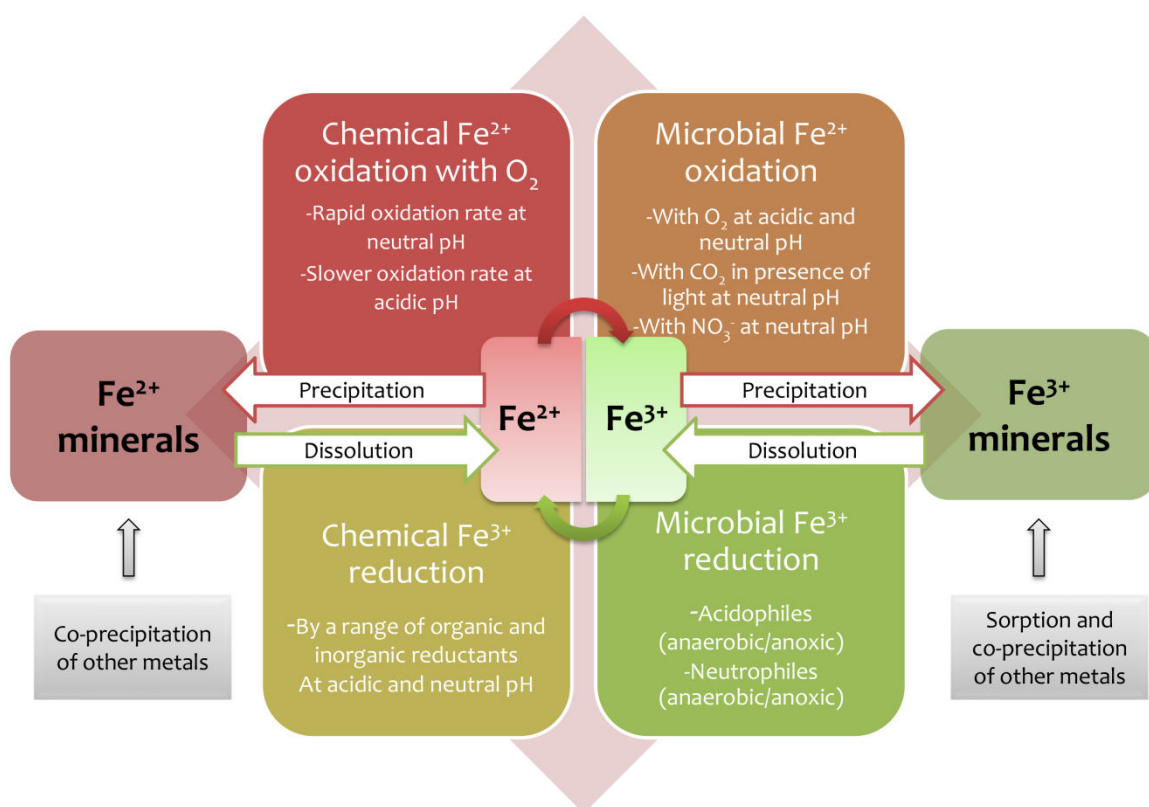


Figure 2.2: Iron biogeochemical cycle

2.2.1 Fe(II) microbial oxidation

The microorganisms capable of oxidizing Fe(II) can be divided into a) aerobic neutrophilic Fe(II)-oxidizing, b) aerobic acidophilic Fe(II)-oxidizing, c) anaerobic Fe(II)-oxidizing phototrophic and d) anaerobic Fe(II)-oxidizing nitrate-reducing bacteria (Kappler and Straub, 2005). All the bacteria of the first three groups belong to the *Proteobacteria* phylum. Ilbert and Bonnefoy (2013) characterized the last group as neutrophilic anaerobes dependent on nitrate, perchlorate or chlorate.

Aerobic neutrophilic Fe(II)-oxidizing microorganisms use oxygen as electron acceptor for enzymatic oxidation of Fe(II). Such bacteria are able to grow under microaerobic conditions and increase the half-life of Fe(II) up to 300 times (Emerson et al., 2010), making biological Fe(II) oxidation predominant over chemical. All the known aerobic neutrophilic iron oxidizers are mesophilic and belong to *Betaproteobacteria* and *Zetaproteobacteria* when isolated from freshwater or marine environments, respectively (Hedrich et al., 2011; Ilbert and Bonnefoy, 2013).

Aerobic acidophilic Fe(II)-oxidizing microorganisms are the most widespread among the Fe(II) oxidizers due to the chemical stability of Fe(II) at acidic pH (Ilbert and Bonnefoy, 2013). They can use solar and chemical sources for energy, a variety of electron acceptors and organic and/or inorganic sources of carbon (Johnson et al., 2012).

The anaerobic phototrophic bacteria grow by using light and Fe(II) as source of energy and electron donor, respectively. Several species have been discovered in freshwater and marine environments (Hedrich et al., 2011). Fe(II) oxidation occurs in a very narrow pH range (between 6.5 and 7) (Kappler and Straub, 2005).

Finally, the first microorganisms capable of oxidizing Fe(II) through dissimilatory reduction of nitrate were discovered about two decades ago (Hafenbradl et al., 1996; Straub et al., 1996). All the intermediates of the nitrate reduction pathway can be used as electron acceptors for Fe(II) oxidation as their redox pair potentials are more positive than that of the couple Fe(III)/Fe(II) at neutral pH. Moreover, this metabolism is more abundant than that by phototrophs as it does not require light (Kappler and Straub, 2005).

To date, the capability to use Fe(II) for energy acquisition has been reported to be widespread among the prokaryotes. Aerobic and anaerobic oxidizers at neutral pH need to deal with the limited availability of dissolved Fe(II) (Konhauser et al., 2011; Ilbert and Bonnefoy, 2013). Moreover, the redox potential of the Fe(II)/Fe(III) couple presents an extreme variability that is dependent on pH and the presence of complexing agents (Ilbert and Bonnefoy, 2013).

2.2.2 Fe(III) reduction

Biological Fe(III) reduction rates depend on the solubility of Fe(III) from mineral dissolution and the affinity with the chelating agents (Bird et al., 2011). Fe(III) is abundant in many environments resulting in a significant variety of Fe(III)-reducing microorganisms which are normally divided into acidophiles and neutrophiles.

Both microbial groups can use reduced sulfur compounds and hydrogen or fermentation products such as simple fatty acids and ethanol as inorganic and organic electron donors, respectively, for Fe(III) reduction (Kappler and Straub, 2005; Johnson et al., 2012). At pH<3, the end product of Fe(II) oxidation by extreme acidophiles is often soluble Fe(III), that can be subsequently used as electron acceptor for Fe(III) reduction by several acidophilic microorganisms (Hedrich et al., 2011). However, up to now the mechanisms of acidophilic bacteria and archaea catalyzing Fe(III) reduction are still poorly understood

(Johnson et al., 2012). At neutral pH, Fe(III) can be a dominant electron acceptor, although its low solubility (Kappler and Straub, 2005; Konhauser et al., 2011) inducing some microorganisms to develop mechanisms to transfer electrons from the cell to the surface of the Fe(III) compounds (Weber et al., 2006a). The preferred sources of solid-phase Fe(III) are amorphous or poorly-ordered iron oxyhydroxides such as ferrihydrite and goethite (Weber et al., 2006a). Nevertheless, microbial Fe(III) reduction has been observed also with Fe(III) oxides with a higher crystallinity (i.e. hematite and magnetite) and Fe-rich clays (e.g. smectite) (Weber et al., 2006a; Konhauser et al., 2011; Kendall et al., 2012).

2.2.3 Formation of Fe minerals

In aquatic environments, Fe minerals can be abiotically and/or biotically produced, with the two typologies of process often occurring simultaneously. Differences in the crystalline structure of the non-biogenic and biogenic minerals have been reported, although the morphology of the crystals is often very similar and requires further advanced mineralogical examination (Chan et al., 2011).

The biological formation of minerals is known as biomineralization and can occur through two main different mechanisms (Konhauser and Riding, 2012). The first mechanism is the “biologically controlled mineralization”, which is independent from the external conditions and mostly controlled by the organisms (Konhauser, 1997; Gadd, 2010). The second mechanism is the ‘biologically induced mineralization’ that intimately occurs depending on the external environmental conditions (Konhauser, 1997; Gadd, 2010).

The most common iron (oxyhydro)oxides on the Earth’s surface are ferrihydrite, goethite, lepidocrocite, hematite, maghemite, magnetite and green rusts. Under aerobic circumneutral conditions, iron exists as amorphous oxyhydroxides and crystalline oxides (Plach et al., 2014). Three general morphologies are known; the cuboidal, parallelepipedal and arrowhead or tooth-shaped crystals. The formed mineral size ranges between 35 and 120 nm (Konhauser, 1997).

Oxidation of dissolved Fe(II) leads to the formation of Fe(III) minerals and the precipitation of various Fe(III) phases. Initially, dissolved Fe(II) species such as $[\text{FeOH}]^{2+}$ and $[\text{Fe}_2(\text{OH}_2)]^{4+}$ are formed during abiotic oxidation. Subsequently, Fe(II) species are transformed into polymeric Fe(III) colloids prior to precipitating as poorly crystalline ferrihydrite particles (2-5 nm as diameter) (Kappler and Straub, 2005). In most cases, ferrihydrite ($\text{Fe}_5\text{HO}_8 \cdot 4\text{H}_2\text{O}$), often written as $\text{Fe}(\text{OH})_3$ or FeOOH , is unstable and tends to be

transformed into more stable iron oxides (crystalline FeOx forms) such as goethite and hematite, depending on the solution composition, temperature and pH (Konhauser, 1997; Dousova et al., 2005; Plach et al., 2014). Ferrihydrite transformation can be either driven by a “solid-state conversion” which is an internal rearrangement of iron and oxygen atoms leading to hematite (Fe_2O_3) formation or by a “dissolution-precipitation mechanism” that results in the production of goethite [$\text{FeO}(\text{OH})$] (Cornell and Schwertmann, 2003).

2.3 Environmental concerns

2.3.1 Iron release and detrimental effects

The maximum acceptable concentration of Fe(II) in drinking water is 0.2 mg/L recommended by the World Health Organization (WHO). The limit of Fe(II) into wastewater varies between 2-5 mg/L for European countries. In water resources, excess iron leads to a reddish color and a distinctive unpleasant smell (Cho, 2015). As a metal, Fe is a non-biodegradable element that can accumulate in the food chain and be adsorbed by living organisms including humans, resulting in serious health disorders, such as depression, rapid and shallow respiration, coma, convulsions, respiratory failure and cardiac arrest according to WHO. Moreover, the presence of iron-utilizing bacteria in Fe(II)-containing water sources can result in infestations of water distribution systems (HDR Engineering Inc., 2002).

Elevated amounts of Fe(II) are contained in several industrial wastewaters such as mining waters and AMD. A mine producing AMD has the potential for long-term devastating impacts on lakes, rivers, streams and aquatic life according to Environmental Law Alliance Worldwide (ELAW). Metal precipitates, particularly iron, coat the stream bottom with an orange-red colored slime giving the water the name of “yellow boy”. The worldwide environmental pollution due to AMD is difficult to evaluate precisely. In 2002, about 240,000 km^2 of Earth's surface were reported to be affected by mining pollution (Furrer et al., 2002). Johnson and Hallberg (2005) estimated that ca. 19,300 km of streams and rivers, and ca. 72,000 ha of lakes and reservoirs worldwide had been seriously damaged by mine effluents. In Canada, there are an estimated 351 million tons of waste rock, 510 million tons of sulfide tailings, and more than 55 million tons of other mining sources which have the potential to cause AMD. Several authors discussed the AMD contamination in the Iberian Pyrite Belt (IPB), one of the largest massive sulfide deposits in the world that is widely known for the intensive mining activity (Sarmiento et al., 2012; Grande et al., 2014). Gold mining also

contributes to iron pollution as gold is intimately associated with sulfide minerals such as pyrite (FeS_2) or arsenopyrite (FeAsS).

2.3.2 Nitrate pollution in the hydrosphere

Nitrate is one of the most common pollutants in water (Bosman, 2009). The regulation of nitrate concentration in drinking water is 50 mg/L for European countries, much higher than in USA where the limit is 10 mg/L. Large amounts of nitrate into water resources have significant ecological and human health effects. For instance, nitrate metabolically reacts with amines and amides and forms nitrosamines, which are known carcinogens (Bosman, 2009). Another international issue associated with nitrate uncontrolled discharge is eutrophication (Camargo et al., 2005).

Nitrogenous compounds are not usually in the list of the ‘chemical concerns’ associated with mining (Bosman, 2009). However, nitrate and ammonium are the most common nitrogen species released to the aquatic environment from mining operations (Zaitsev et al., 2008). This is mainly due to the use of extensive amounts of explosives such as ‘ammonium nitrate fuel oil’ (ANFO) or leaching solutions to elute elements such as copper, nickel, gold and uranium (Koren et al., 2000; Zaistev et al., 2008; Hayrynen et al., 2009).

Nitrate is stable and highly soluble with low potential for co-precipitation or adsorption (Luk and Au-Yeung, 2002). Various technologies are available for nitrate treatment, such as ion exchange, adsorption, chemical treatment, and membrane technology (Park and Yoo, 2009). Biologically, denitrification for nitrate removal is a well-established technology which is feasible even under low pH and temperature conditions and in presence of toxic metals (Papirio et al., 2014b).

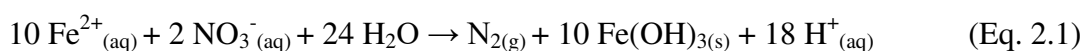
2.4 Fe(II) iron-mediated autotrophic denitrification

Recently, biotechnologies have been largely used for the recovery of metals and involve the development of technologies based on the understanding of living organisms (Fig. 2.3). Fe(II) iron-mediated autotrophic denitrification is a new bioprocess for removal/recovery of iron and reduction of nitrate from wastewaters. Anaerobic nitrate-dependent Fe(II) oxidation has most likely evolved in the primordial deep sea water that was rich in NO_3^- due to the N_2 conversion during lightning discharge on the early Earth (Ilbert

and Bonnefoy, 2013). Despite this, biological nitrate-dependent Fe(II) oxidation was only discovered 20 years ago by Straub et al. (1996). This process is based on the bio-oxidation of Fe(II) to Fe(III), followed by the bioprecipitation of Fe(III) (hydr)oxides with possible co-precipitation or adsorption of other metals. Electrons are transferred to nitrate that is anaerobically reduced to nitrogen gas through denitrification (Devlin et al., 2000). Complete nitrate reduction to N₂ without accumulation of intermediates has been reported in most studies (Straub et al., 1996; Chaudhuri et al., 2001; Weber et al., 2006b; Blothe and Roden, 2009). However, depending on the microbial culture used, a significant production of nitrite and nitrous oxide has been observed (Li et al., 2014).

Recently, Fe(II)-mediated autotrophic denitrification is gaining scientific interest over classical heterotrophic denitrification especially for the treatment of industrial wastewaters. Such waters are usually poor of carbon and the supplementation of organics in order to stimulate heterotrophic denitrification would result in secondary organic pollution and additional treatment and operating costs (Zhang et al., 2015). Moreover, autotrophic microorganisms consume larger quantities of energy-generating substances for the reduction of CO₂, inducing lower biomass production and potential for biofouling in comparison with heterotrophic denitrifiers (Devlin et al., 2000). However, autotrophic bacteria growth is generally slower because of the incomplete Krebs cycle that generates less ATP (Mattes et al., 2013).

Besides Fe(II), autotrophic denitrifiers can use other inorganic electron donors, such as zero-valent iron (Biswas and Bose, 2005), pyrite (FeS₂) (Bosch et al., 2012), hydrogen gas H₂ (Chen et al., 2014), reduced sulfur compounds (Zou et al., 2016) and As(III) (Senn and Hemond, 2002) assimilating dissolved CO₂ (or HCO₃⁻) as source of carbon for microbial cell synthesis (Straub et al., 1996). Results showed that the performance of Fe(II)-mediated autotrophic denitrification is fairly higher than denitrification with other electron donors such as sulfur and hydrogen (Park and Yoo, 2009; Zhang et al., 2015). Also, in presence of metals such as Cu(II) and Ag(I), Fe(II)-driven denitrification is catalyzed resulting in higher nitrate removal rates and the possibility of recovering iron and other metals (Hansen et al., 1996). The complete reduction of nitrate to dinitrogen gas is as suggested by Sorensen (1987):



The mechanisms used by the neutrophilic nitrate-dependent Fe(II) oxidizers are, however, not well defined yet. An energetic benefit from this metabolism for the organisms involved is achieved at circumneutral and alkaline pH (Muehe et al., 2009; Weber et al., 2009). Under these conditions, the redox potential of the Fe(II)/Fe(III) couple (+200 mV) is lower than the $\text{NO}_3^-/\text{NO}_2^-$ couple (+430 mV) in standard conditions promoting the reaction between Fe(II) and NO_3^- . In contrast, the redox potential of the Fe(II)/Fe(III) couple is much higher (+770 mV) and the reaction is inhibited at acidic pH (Hedrich et al., 2011).

2.4.1 Fe(II)- oxidizing denitrifiers

Nitrate-dependent Fe(II) oxidizers are widespread among the prokaryotes and responsible for dissimilatory Fe(II) metabolism (Ilbert and Bonnefoy, 2013). There are even evidences that *Escherichia coli*, a well-known anaerobic bacterium, is capable of Fe(II) oxidation coupled to nitrate reduction (Brons et al., 1991; Carlson et al., 2012; Carlson et al., 2013). Although Fe(II) is used as main electron donor, most of the microbial species capable of this metabolism do not grow under strict lithotrophic conditions but require an additional electron donor or organic carbon as energy source (Straub et al., 2004). Nitrate-dependent Fe(II) oxidation has been observed with both mixed enrichments and pure cultures. Tables 2.1 and 2.2 report the prokaryotes capable of maintaining nitrate dependent Fe(II) oxidation under mixotrophic and strictly autotrophic conditions, respectively.

Microorganisms have been isolated from various habitats, such as freshwater and saline environments, paddy soils, ponds, streams, ditches, brackish lagoons, lake sediments, wetlands, aquifers, hydrothermal and deep-sea sediments (Weber et al., 2006a; Hedrich et al., 2011). The various microorganisms mostly belong to archaeal and eubacterial genera of the phylum *Proteobacteria* (classes α -, β -, γ - and δ -) (Hedrich et al., 2011). The majority of the studies has been performed under mesophilic conditions (30-40°C) or at room temperature, as most of the microorganisms capable for the process are mesophilic. However, both psychrophilic and thermophilic organisms are known, as the bacterium *Thermomonas* strain BrG3, that grows in the range 4-37°C (Mergaert et al., 2003), and the hyperthermophilic archaeum *Ferroglobus placidus* that has an optimal growth at 85°C (Hafendradl et al., 1996).

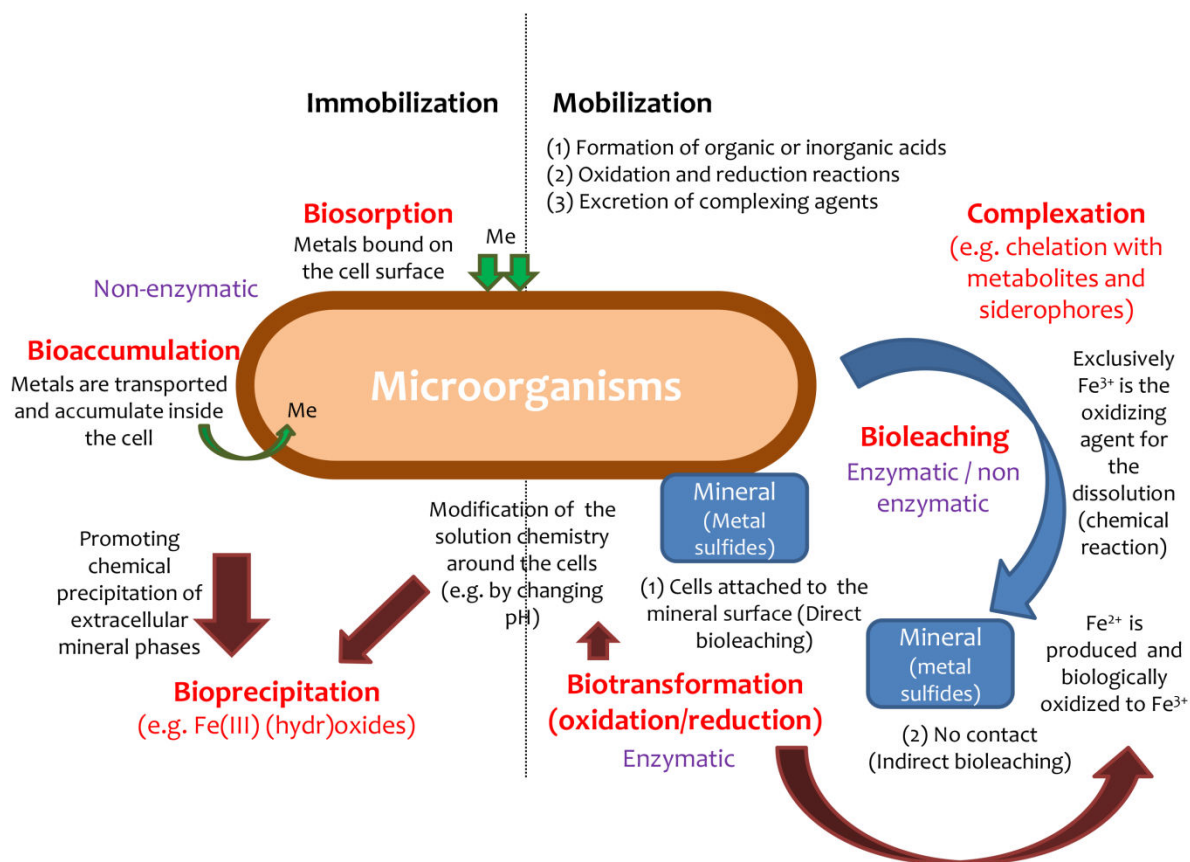


Figure 2.3: Schematic illustration of the main mechanisms used by microbes to approach to metals for metal biorecovery

2.4.1.1 Mixed cultures

The ability to oxidize Fe(II) with nitrate is widespread amongst the *Proteobacteria* and may also be found among the Gram-positive bacteria with high guanine-cytosine (GC) content of DNA (Straub et al., 2004). Nitrate-dependent Fe(II) oxidation has been reported by enrichment cultures mostly collected from freshwater and brackish water sediments. Most cultures are mixotrophic and need an organic carbon source in order to oxidize Fe(II) and reduce nitrate (Straub et al., 1996; Benz et al., 1998). Only few cultures are capable of a strict autotrophic metabolism such as freshwater enrichment KS (Straub et al., 1996) and an enrichment culture from marine sediment (Benz et al., 1998). The capability of maintaining Fe(II) -driven denitrification by the autotrophic freshwater enrichment culture KS has been thoroughly demonstrated (Buchholz-Cleven et al., 1997; Schaedler et al., 2009; Weber et al., 2001; Blothe and Roden, 2009; Hohmann et al., 2009; Shelobolina et al., 2012). Furthermore, the culture was shown to be capable of nitrate-dependent oxidation of Fe(II) solid-phases

such as biotite (Weber et al., 2001) and Fe(II)-rich trioctahedral mica in granitic rocks (Shelobolina et al., 2012).

Fe(II)-driven autotrophic denitrification by traditional activated sludge was observed by Nielsen and Nielsen (1998) for the first time in wastewater treatment plants aimed at biological phosphorus removal. In presence of autotrophic denitrifiers, Fe(II) supplementation results in Fe(II) oxidation to Fe(III) that promotes chemical phosphorus removal and enhances sludge flocculation properties. A heterotrophic denitrifying enriched culture from a lab-scale high-rate denitrifying reactor was able to perform nitrate-dependent anaerobic Fe(II) oxidation (Wang et al., 2014). The same culture was used for the bioaugmentation of ongoing high efficiency nitrate-dependent Fe(II) oxidation reactors (Wang et al., 2015).

Mixed cultures are generally preferred in environments contaminated by several substances, such as other metals, arsenic, uranium and hydrocarbons. Anaerobic oxidation of Fe(II) dependent on nitrate has been observed in toluene-degrading incubations, petroleum-contaminated aquifer sediments and sludge from oil water separators (Caldwell et al., 1999). Hydrocarbon and Fe(II) oxidation simultaneously occurred with nitrate as electron acceptor. Fe(II) oxidation was enhanced when organic electron donors were provided (Caldwell et al., 1999). The supplementation of nitrate in aquifer sediments contaminated with uranium stimulated Fe(II) and U(IV) oxidation (Finneran et al., 2002). Anaerobic As(III) and Fe(II) oxidation can be simultaneously performed with autotrophic denitrification with lake (Senn and Hemond, 2002) and activated sludge communities (Sun et al., 2009a). The use of microorganisms belonging to the genus *Azoarcus* and the family *Comamonadaceae* resulted in the removal of As(V) through immobilization of As(V) onto Fe(III) (hydr)oxides (Sun et al., 2009a).

In mixed cultures, chemolithotrophic Fe(II)-oxidizing denitrifiers are usually present in a lower amount than other species, not however affecting the ability of oxidizing Fe(II) (Straub and Buchholz-Cleven, 1998). The enumeration and detection of nitrate-dependent Fe(II)-oxidizing bacteria in different freshwater sediments showed that the number of mixotrophic nitrate-reducing iron oxidizers is two order of magnitude lower than that of the heterotrophic (acetate)-oxidizing denitrifiers (Muehe et al., 2009). Quantifications by most probable number showed the presence of 1×10^4 autotrophic and 1×10^7 heterotrophic nitrate-dependent Fe(II) oxidizers per gram of fresh sediment in a group of uncultured *Actinobacteria* (Kanaparthi et al., 2013).

Table 2.1: Prokaryotes capable of maintaining nitrate dependent Fe(II) oxidation under mixotrophic (low amount of organic substrate, 0.1-1.5 mM) conditions.

Species	Phylum (PNU), Class, Family	Isolation site	Growth pH	Growth T(°C)	Electron Donor (Fe ²⁺)	Details	References
Denitrifying bacterial strain LP-1 and strain AR-1	-	Activated sludge of municipal sewage plant	7	15-28	FeSO ₄	Addition of 1 mM acetate, tentatively classified within the family <i>Pseudomonadaceae</i>	Gallus and Schink, 1994; Straub et al., 1996
Denitrifying bacterial strain HidR2	–	Brackish water lagoon	6.7-7	15-30	FeSO ₄	Presence of acetate (≤ 1 mM)	Straub et al., 1996; Benz et al., 1998
<i>Acidovorax</i> strain BrG1	<i>B-Proteobacteria</i> , Class β, <i>Comamonadaceae</i>	Town ditches, freshwater sediments	6-7	15-30	FeS, FeSO ₄	Presence of acetate (≤ 1 mM)	Straub et al., 1996; Buchholz-Cleven et al., 1997; Straub and Buchholz, 1998; Straub et al., 2004; Haaijer et al., 2012
<i>Aquabacterium</i> strain BrG2	<i>Proteobacteria</i> , Class β, <i>Comamonadaceae</i>	Town ditches, freshwater sediments	6-7	15-30	FeS, FeSO ₄	Addition of 0.5 mM fumarate, Fe(II) oxidation in 1 week	Straub et al., 1996; Buchholz-Cleven et al., 1997; Straub and Buchholz, 1998; Straub et al., 2004
<i>Thermomonas</i> strain BrG3	<i>Proteobacteria</i> , Class γ, <i>Xanthomonadaceae</i>	Freshwater sediments	6-7	4-37, 27-37(op)	FeSO ₄	Presence of low concentrations of acetate	Straub et al., 1996; Buchholz-Cleven et al., 1997; Straub and Buchholz, 1998
<i>Acidovorax</i> strain BoFeN1	<i>Proteobacteria</i> , Class β, <i>Comamonadaceae</i>	Lake and freshwater sediments	6-9, 7-7.5 (opt.)	4-37, 30(opt.)	FeSO ₄ , FeCl ₂ , vivianite [Fe ^{II} ₃ (PO ₄) ₂ ·8H ₂ O]	Requires 0.2-1 mM for 4 mM of dissolved Fe(II).	Kappler, et al., 2005; Muehe et al., 2009; Shaedler et al., 2009; Miot et al., 2009a, 2014; Kappler et al., 2010; Hohmann et al., 2009; Larese-Casanova et al., 2010; Pantke et al., 2012; Klueglein and Kappler, 2013; Schmid et al., 2014; Klueglein et al., 2015

<i>Acidovorax</i> strain 2AN	<i>Proteobacteria</i> , Class β , <i>Comamonadaceae</i>	Mixture of iron-oxide-bearing sediments	circumn eutral, 6.8-6.9	30	FeCl ₂ , Fe(II)-EDTA, solid-phase Fe(II)	Addition of acetate	Chakraborty et al., 2011; Chakraborty and Picardal, 2013a
<i>Acidovorax ebreus</i> strain TPSY (formerly <i>Diaphorobacter</i> sp. strain TPSY)	<i>Proteobacteria</i> , Class β , <i>Comamonadaceae</i>	Groundwater	4.5-9, 7.25 (opt.)	20-37, 37 (opt.)	FeCl ₂	Closely related to <i>Diaphorobacter nitroreducens</i> . Addition of 0.1 mM acetate	Byrne-Bailey et al., 2010; Zhu et al., 2013
<i>Dechloromonas</i> sp. strain UWNR4	<i>Proteobacteria</i> , Class β , <i>Rhodocyclaceae</i>	River sediments	6.8-7	30	FeCl ₂ , Fe(II)-EDTA, solid-phase Fe(II)	(Per)chlorate reducing bacterium, addition of 0.5 mM acetate	Coby et al., 2011; Chakraborty and Picardal, 2013a;b

Table 2.2: Prokaryotes capable of maintaining nitrate dependent Fe(II) oxidation under mixotrophic (low amount of organic substrate, 0.1-1.5 mM) conditions.

Species	Phylum (PNU), Class, Family	Isolation site	Growth pH	Growth T(°C)	Electron Donor (Fe ²⁺)	Details	References
Denitrifying bacterial strain ToN1	-	Freshwater mud samples	7	15-28	FeS, FeSO ₄	Close relationship to <i>Thauera selenatis</i>	Rabus and Widdel, 1995; Straub et al., 1996
<i>Pseudomonas stutzeri</i>	<i>Proteobacteria</i> , Class γ , <i>Pseudomonadaceae</i>	Soil and marine environments	7	15-28	FeSO ₄		Straub et al., 1996; Muehe et al., 2009
<i>Pseudomonas</i> sp. SZF15	<i>Proteobacteria</i> , Class γ , <i>Pseudomonadaceae</i>	Reservoir sediments	6.5	30	FeCl ₂	Similarity (100%) to <i>Pseudomonas</i> sp. (NR037135)	Su et al., 2015
<i>Paracoccus denitrificans</i>	<i>Proteobacteria</i> , Class α , <i>Rhodobacteraceae</i>	Soil (aerobic and anaerobic environments)	7	28	FeSO ₄ , Fe(II)-EDTA		Kumaraswamy et al., 2006; Muehe et al., 2009
<i>Paracoccus ferrooxidans</i> strain BDN-1	<i>Proteobacteria</i> , Class α , <i>Rhodobacteraceae</i>	Denitrifying bioreactor (full-scale FBR that treated surface water)	5-8.5, 7 (opt.)	10-45, 30 (opt.)	Fe(II)-EDTA	Similarity 99.29% to <i>P. denitrificans</i>	Kumaraswamy et al., 2006

<i>Ferroglobus placidus</i>	Archaeum, <i>Archaeoglobi</i> , <i>Archaeoglobaceae</i>	Shallow submarine hydrothermal system at volcano	6-8.5, 7 (opt.)	65-95, 85 (opt.)	FeS, FeCO ₃	Hyperthermophilic	Hafenbradl et al., 1996
Iron-oxidizing bacteria (FeOB) strains F01, F02, F03	<i>Proteobacteria</i> , Class α , <i>Hyphomonadaceae</i>	Mineral surface,		30		<i>Hyphomonas jannaschiana</i> (81 to 89% similarity)	Edwards et al., 2003
Iron-oxidizing bacteria (FeOB) strains F04, F05, F06, F08, F08, F09, F015	<i>Proteobacteria</i> , Class γ , <i>Alteromonadaceae</i>	Seafloor near active hydrothermal fields	7	maximum, 3-10 (opt.)	FeCO ₃ , FeCl ₂	<i>Marinobacter aquaeolei</i> (87-94% similarity)	
<i>Azospira</i> bacterium TR1	<i>Proteobacteria</i> , Class β , <i>Rhodocyclaceae</i>	Working bioremediation site	7	Room T	FeSO ₄		Mattes et al., 2013
<i>Citrobacter freundii</i> strain PXL1	<i>Proteobacteria</i> , Class γ , <i>Enterobacteriaceae</i>	Anoxic activated sludge of sewage plant	7	30	FeCl ₂ 4H ₂ O	In AsO ₄ ³⁻ 2.85-13.65 mM, efficiently oxidized Fe(II), reduce nitrate and remove As(III) (Li et al., 2015)	Li et al., 2014, 2015
<i>Thiobacillus</i> <i>denitrificans</i>	<i>Proteobacteria</i> , Class β , <i>Hydrogenophilaceae</i>	Soil, mud, freshwater and marine sediments, sewage industrial wastewater treatment ponds, digestion tanks	1.6, 6.8- 7.4, 6.9 (op)	28-32, 29.5 (op)	FeCO ₃ , FeS, FeS ₂ , FeSO ₄ , FeCl ₂	Requires the addition of extra electron donors	Straub, et al., 1996; Kelly and Wood, 2000; Haaijer et al., 2006; Beller et al. 2006; Kanaparthi and Conrad, 2015
<i>Pseudogulbenkiania</i> <i>ferrooxidans</i> strain 2002 (formerly <i>Lutiella</i> <i>nitroferrum</i> strain 2002)	<i>Proteobacteria</i> , Class β , <i>Neisseriaceae</i>	Freshwater lake sediments	6.75-8	15-40	FeCl ₂ , nontronite (NAu-2)	Similarity 99.8% to <i>Pseudogulbenkiania</i> sp. strain NH8B	Weber et al., 2006b, 2009; Tago et al., 2011; Byrne-Bailey et al., 2012; Zhao et al., 2013; 2015
<i>Candidatus Kuenina</i> <i>stuttgartiensis</i>	Anammox, Taxonomical Status: <i>Candidatus</i> , genus: <i>Kuenina</i>	Sludge from nitrification stage of wastewater treatment plant	8	—	Fe ²⁺		Strous et al., 2006
<i>Candidatus Brocadia</i> <i>sinica</i>	Anammox, Taxonomical Status: <i>Candidatus</i> , genus: <i>Brocadia</i>	Activated sludge from a municipal wastewater treatment plant	5.9-9.8 (not below 5)	25-37, 30-37 (opt.)	FeSO ₄		Oshiki et al., 2013

The activity of chemolithotrophic Fe(II) oxidizing denitrifiers can be reduced by the presence of phototrophic microorganisms using Fe(II) as electron donor in freshwater lake ecosystems. Phototrophs have been shown to oxidize Fe(II) more quickly and potentially outcompete autotrophic denitrifiers under optimal light conditions. A diel cycle is needed in order to promote chemolithotrophic Fe(II)-mediated denitrification (Melton et al., 2012).

2.4.1.2 Autotrophic and mixotrophic growth in pure cultures

Several pure cultures of Fe(II) oxidizers have been isolated and tested for Fe(II)-mediated denitrification under both mixotrophic and strict autotrophic conditions. Most of these microorganisms have only been used in a few studies and are here briefly described. The most studied bacteria are instead reported in the following subsections and analyzed in detail. The strain ToN1, intimately close to *Thauera selenatis*, as well as *P. stutzeri* and the strain SZF15, belonging to the *Pseudomonas* genus, have been demonstrated to be capable of denitrifying by using Fe(II) as sole electron donor (Straub et al., 1996; Muehe et al., 2009; Su et al., 2015). *Paracoccus denitrificans* and *ferrooxidans* strains are facultative autotrophs and can perform Fe(II) oxidation with uncomplexed Fe(II) and $[\text{Fe(II)EDTA}]^{2-}$ (Kumaraswamy et al., 2006; Muehe et al., 2009). A novel anaerobic autotrophic nitrate-dependent Fe(II) oxidizer, the strain *Citrobacter freundii* PXL1, was isolated from an anoxic sludge of a wastewater treatment plant and reported to have the potential of synchronous removal of nitrate, Fe(II) and arsenite (Li et al., 2014; 2015).

Gallionella ferruginea is also related with autotrophic nitrate-dependent Fe(II) oxidation as reported in the studies of Gouy et al. (1984) and Hallbeck et al. (1993). 16S rRNA gene libraries from the enrichment culture KS were dominated by a phylotype related to *Sideroxydans lithotrophicus* and *Gallionella ferruginea* by 95 and 94%, respectively (Blothe and Roden, 2009). Organisms related to *Gallionella* and *Sideroxydans* species were labeled in the autotrophic nitrate-dependent Fe(II)-oxidizing culture TM3 group of uncultured *Actinobacteria* (Kanaparthi et al., 2013).

Fe(II)-oxidizing denitrifiers have also been found in extreme environments. The neutrophilic archaeum *Ferroglobus placidus* was isolated from a shallow submarine hydrothermal system, growing between 65 and 95°C (Hafenbradl et al., 1996). Under psychrophilic conditions (3 to 10°C), chemolithotrophic nitrate dependent iron-oxidizing bacteria were observed on the seafloor near an active hydrothermal field and capable of oxidizing different Fe(II) sources (FeS, FeCO₃, FeCl₂ and FeS₂) (Edwards et al., 2003).

Many cultures are instead reported to require a source of organic carbon as an additional electron donor. The strain HidR2, isolated from brackish-water sediments, and the strains LP-1 and AR-1, originally observed in an activated sludge, are able to anaerobically oxidize Fe(II) only in presence of 0.2-1.1 mM of acetate (Straub et al., 1996; Benz et al. 1998). LP-1 was tentatively classified within the family of *Pseudomonadaceae* (Gallus and Schink, 1994). Other cultures require larger amount of organic carbon (>1.5 mM) for mixotrophic growth, such as the strain FW33AN (99% 16S rDNA sequence identity with *Klebsiella oxytoca*) that was isolated from an U(VI)- and NO₃⁻ contaminated groundwater (Senko et al., 2005a, 2005b). In agreement, the ability of *Klebsiella* species for nitrate reduction was also reported in experiments with the bacterium *K. pneumoniae* L17 with glucose and Fe(II) as electron donors (Liu et al., 2014). The strain *Desulfitobacterium frappieri* G2 requires acetate and Fe(II) in equivalent amount to oxidize soluble and structural Fe(II) and reduce nitrate (Shelobolina et al., 2003). No growth in the absence of either Fe(II) or acetate is observed. A pure culture of *Geobacter metallireducens* is able to oxidize both Fe(II) and U(IV) in presence of acetate with nitrate as electron acceptor (Finneran et al., 2002).

Pure cultures isolated from the freshwater enrichment KS

Three strains BrG1, BrG2 and BrG3 were isolated from the strictly lithotrophic culture KS and shown to be responsible for Fe(II)-mediated autotrophic denitrification (Straub et al., 1996). Only BrG2 is capable of autotrophically oxidizing Fe(II), while BrG1 and BrG3 cometabolize Fe(II) with 0.5-1 mM of acetate as an additional electron donor and carbon source (Straub et al., 1996; Straub and Buchholz-Cleven, 1998). BrG1 and BrG2 strains belong to β -*Proteobacteria* (Buchholz-Cleven et al., 1997). The BrG3 strain was affiliated with the *Xanthomonas* group of the γ -*Proteobacteria*. Straub et al. (2004) also reported that strains BrG1, BrG2 and BrG3 are affiliated with the genera *Acidovorax*, *Aquabacterium*, and *Thermomonas*, respectively. This has also been confirmed by Mergaert et al. (2003) who isolated a new species, named *Thermomonas fusca*, belonging to the genus *Thermomonas*, with a 99.6% similarity to the strain BrG3. *Acidovorax* strain BrG1 can be abundant in iron sulfide mineral and nitrate-rich groundwater ecosystems (Haaijer et al., 2012).

Thiobacillus denitrificans

Thiobacillus denitrificans is a well-known bacterium that was first isolated by Beijerinck in 1904. *T. denitrificans* can be found in soil, mud, freshwater, marine sediments and also in domestic sewage and industrial wastewater treatment ponds, especially under anoxic conditions. *T. denitrificans* grows as an anaerobic chemolithotroph on thiosulfate, tetrathionate, thiocyanate, sulfide or elemental sulfur by using nitrate, nitrite or nitrous oxide as terminal respiratory oxidants (Kelly and Wood, 2000; Zou et al., 2016). Nonetheless, also Fe(II) has been observed to promote *T. denitrificans* growth under strictly autotrophic conditions (Straub et al., 1996). Supplementation of Fe(II) as FeS or FeS₂ is preferable as sulfur acts as energy source. Beller et al. (2006) reported the complete genome sequence of *T. denitrificans* strain ATCC 25259 and its capability for nitrate-dependent oxidation of Fe(II) added as siderite (FeCO₃). On the contrary, *T. denitrificans* has been reported to be unable to oxidize Fe(II) in the form of FeSO₄ (Muehe et al., 2009).

In contrast with the previous studies, Kanaparthi and Conrad (2015) found that a pure culture of *T. denitrificans* was incapable to maintain chemolithotrophic nitrate-dependent Fe(II) oxidation without the presence of humic substances. Humic substances significantly enhanced the bacterial growth and activity, probably due to the chelation of iron, while addition of other chelating agents such EDTA inhibited the growth of *T. denitrificans* (Kanaparthi and Conrad 2015).

Pseudogulbenkiania ferrooxidans sp. strain 2002

Pseudogulbenkiania ferrooxidans strain 2002 (formerly known as *Lutiella nitroferum*) was isolated from a freshwater lake sediment (Weber et al., 2006b) and commonly found as dominating species in paddy soils used for the cultivation of rice and soybean (Tago et al., 2011). This novel strain of nitrate-dependent Fe(II) oxidizing microorganisms belongs to the family of *Neisseriaceae* in the β -class of *Proteobacteria* (Weber et al., 2006b, 2009; Byrne-Bailey et al., 2012). *P. ferrooxidans* is an anaerobic (nitrate-dependent), mesophilic, neutrophilic Fe(II)-oxidizing lithotroph which does not need addition of organic substrate. Soluble Fe(II) is used as sole electron donor with nitrate reduced to gaseous end products (N₂ or N₂O) by a strict lithotrophic metabolism. *P. ferrooxidans* is also capable of oxidizing Fe(II) in the clay mineral nontronite (NAu-2) (Zhao et al., 2013, 2015).

Species of the genus *Acidovorax*

The genus *Acidovorax* is associated with nitrate-dependent Fe(II) oxidation, as four *Acidovorax* species within the class of β -*Proteobacteria* are capable of performing Fe(II)-driven denitrification. The strains are known as BrG1, BoFeN1, 2AN and *Ebreus* strain TPSY.

The *Acidovorax* strain BoFeN1 was isolated from a freshwater lake sediment (Kappler et al., 2005) and can only grow mixotrophically with acetate concentrations ranging between 0.3 and 5 mM (Kappler et al., 2005; Muehe et al., 2009; Pantke et al., 2012; Klueglein and Kappler, 2013; Miot et al., 2014). The *Acidovorax* strain BoFeN1 is capable to oxidize Fe(II) in soluble and mineral forms (i.e. vivianite) (Larese-Casanova et al., 2010; Miot et al., 2009a). In comparison with other pure cultures, i.e. *P. stutzeri* and *P. denitrificans*, *Rhodobacter ferrooxidans* strain SW2 and the mixed culture KS, *Acidovorax* sp. strain BoFeN1 results in higher Fe(II) oxidation rates (Muehe et al., 2009; Hohmann et al., 2009).

The *Acidovorax* strain 2AN was enriched from a mixture of iron-oxide-bearing sediments (Chakraborty et al., 2011). This species is able to oxidize aqueous non-complexed Fe(II), chelated Fe(II) (i.e. Fe(II)-EDTA), and solid-phase Fe(II) coupled to nitrate reduction at circumneutral pH, in presence of acetate (Chakraborty and Picardal, 2013a).

The nitrate-dependent Fe(II) oxidizing strain TPSY was isolated from groundwater as a facultative anaerobe, capable of mixotrophic growth on Fe(II) and nitrate with acetate (Zhu et al., 2013; Carlson et al., 2013). This strain has been renamed *Acidovorax ebreus* and is closely related to *Diaphorobacter nitroreducens* (Byrne-Bailey et al., 2010), capable of oxidizing Fe(II)-NTA (nitrilotriacetic acid), Fe(II)-NTA-agarose and synthetic vivianite in presence of acetate (Carlson et al., 2013).

Perchlorate-reducing bacteria

Several studies reported the ability of perchlorate-reducing bacteria for nitrate-dependent Fe(II)-oxidation (Weber et al., 2006c; Coby et al., 2011; Chakraborty and Picardal, 2013a;b). The dominating bacteria detected in freshwater sediments responsible for the process are all β -*Proteobacteria* *Dechloromonas* species (Weber et al., 2006c; Coby et al., 2011). The *Dechloromonas* strain UWNR4 is able to oxidize both soluble Fe(II) and chelated Fe(II)-EDTA with acetate (Coby et al., 2011; Chakraborty and Picardal, 2013a;b). The *Dechlorosoma suilium* strain PS has also been shown to perform Fe(II)-mediated

denitrification under mixotrophic conditions (Chaudhuri et al, 2001; Lack et al., 2002a). *D. suillum* strain PS has 99% similarity to the endophyte *Azospira oryzae* strain N1 found in several species of rice (Reinhold-Hurek and Hurek, 2000; Byrne-Bailey and Coates, 2012) and in rice paddy soils (Ratering and Schnell, 2001; Tago, 2011).

Anammox bacteria

Anaerobic ammonium oxidizing (anammox) bacteria are capable of maintaining nitrate-dependent Fe(II) oxidation. Investigations on iron and manganese respiration by *Candidatus Kuenina stuttgartiensis* indicated that iron was oxidized with nitrate as electron acceptor (Strous et al., 2006). Also enrichment cultures of *Candidatus Brocadia sinica* and *Candidatus Scalindua sp.* anaerobically oxidize Fe(II) while reducing NO_3^- to nitrogen gas (Oshiki et al., 2013). Moreover, the supplementation of Fe(II) has been observed to be stimulatory for anammox bacteria enhancing the specific growth rates that are generally rather low (Liu and Ni, 2015).

2.4.2 Operating parameters affecting Fe(II)-mediated autotrophic denitrification

The studies that investigated Fe(II)-mediated autotrophic denitrification can be divided into those focusing on 1) nitrate removal and 2) iron biorecovery with characterization of Fe(III) precipitates. Most of the studies have been performed within batch systems, whereas only a few works evaluated the potential of continuous-flow reactors (Sun et al, 2009b; Chakraborty et al., 2011; Wang et al., 2015; Zhang et al., 2015) and were recently reviewed (Di Capua et al., 2015). In the following sections, the impacts of the operating parameters on Fe(II)-driven denitrification are illustrated.

2.4.2.1 pH

pH is a very important parameter affecting both microbial activity and iron speciation. At very acidic pH, Fe(II) is stable but biological activity of most denitrifying cultures is repressed. On the other hand, at circumneutral pH the denitrifying biological activity is favored but Fe(II) is more rapidly oxidized. Most of the studies were performed with a feed pH ranging between 6.0 and 8.0, as none of the known Fe(II)-oxidizing denitrifiers has been reported to be acidophilic. However, the TM3 group of uncultured *Actinobacteria* was found to be capable of performing the process at pH 4.5 (Kanaparthi et al., 2013).

As Fe(II)-mediated autotrophic denitrification produces acidity, a feed pH higher than 7.0 is generally recommended in order to strengthen the process (Zhang et al., 2015). An investigation of the optimal pH showed that nitrate-dependent Fe(II) oxidation occurred with the highest rates and efficiency at pH between 6.4 and 6.7 (Straub et al., 2004). In continuous flow experiments, nitrate removal was higher than 95% as long as the effluent pH was higher than 6.0 (Zhang et al., 2015). Oshiki et al. (2013) reported a wider optimal pH range (between 5.9 and 9.8) and did not observe Fe(II) oxidation and NO_3^- reduction at pH below 5.0. Larese-Casanova et al. (2010) tested three different pH values (6.3, 7.0, 7.7) and observed significant differences in Fe(III) mineralogy although the small pH range. At pH 6.3, only lepidocrocite was observed, whereas an increasing amount of goethite was detected at higher pH.

2.4.2.2 Temperature

Most of the studies on Fe(II)-driven autotrophic denitrification was carried out under mesophilic conditions as temperatures in the range 28-40°C are optimal for the growth of Fe(II)-oxidizing denitrifiers (Straub et al., 1996; Oshiki et al., 2013). Microcosms prepared under psychrophilic, thermophilic, and hyperthermophilic conditions in the range of 4-75°C resulted in lower microbial activities and denitrification rates (Oshiki et al., 2013).

2.4.2.3 Fe(II)/ NO_3^- ratio

A molar Fe(II)/ NO_3^- ratio of 5 has been observed as the most effective to achieve a complete and faster nitrate removal with different microbial cultures and operating conditions (Straub et al., 1996; Blothe and Roden, 2009; Chaudhuri et al., 2001; Weber et al., 2006b). At Fe(II)/ NO_3^- ratios lower than 4.0, a significant production of intermediates such as nitrite and nitrous oxide has been observed (Li et al., 2014). On the contrary, Fe(II)/ NO_3^- ratios higher than 7.5 result in dissimilatory nitrate reduction to ammonium and, thus, remaining nitrogen pollution (Li et al., 2014). When formed during denitrification, nitrite can chemically react with Fe(II) (Shelobolina et al., 2003; Kumaraswamy et al., 2006; Larese-Casanova et al., 2010; Klueglein and Kappler, 2013; Zhao et al., 2013) resulting in the production of Fe(III) and reduced nitrogen compounds including NO, N_2O , and N_2 (Shelobolina et al., 2003; Kumaraswamy et al., 2006). Chemical oxidation of Fe(II) with nitrite occurs only when nitrite concentration is higher than 1 mM (Straub et al., 1996), and results in lower oxidation

rates than microbial Fe(II) oxidation (Kappler et al., 2005). Depending on the microbial culture, a lower Fe(II)/NO₃⁻ ratio than 5 can be used. Oshiki et al., (2013) observed that two pure anammox enrichment cultures *Candidatus Brocadia sinica* and *Candidatus Scalindua* required 2.8 and 3.3 moles of Fe(II) to reduce 1 mol of NO₃⁻, respectively.

2.4.2.4 Fe(II) and NO₃⁻ feed concentrations and sources

The average Fe(II) and NO₃⁻ concentrations used in most studies are in the ranges 2.0-10.0 and 0.1-6.0 mM, respectively. However, higher feed Fe(II) concentrations have been tested. Feed Fe(II) and NO₃⁻ as high as 25 and 10 mM, respectively, did not inhibit the growth of *Dechlorosoma suillum* (Lack et al., 2002a). Similarly, the activity of *Candidatus Brocadia sinica* and *Candidatus Scalindua* was not affected in presence of 15 mM of Fe(II) (Oshiki et al., 2013).

Higher concentrations of NO₃⁻ have also been tested, without any inhibitory effect (Liu et al., 2014). Concentrations between 5.8-8.0 mM of NO₃⁻ and 1.0-5.0 mM of Fe(II) resulted in 30-45% of nitrate removal and 85-100% of Fe(II) oxidation in 3 to 6 d by a pure culture of *Citrobacter freundii* strain PXL1.

Fe(II) has been mostly supplemented as FeCl₂, FeSO₄, FeCO₃, FeS and Fe(NH₄)₂(SO₄) (Caldwell et al., 1999; Hafenbradl et al., 1996; Straub et al., 1996; Weber et al., 2006b). Minerals such vivianite (Fe(II)₃(PO₄)₂·8H₂O), nontronite (Na_{0.3}Fe₂((Si,Al)₄O₁₀)(OH)₂·nH₂O), and biotite K(Fe)₃(AlSi₃O₁₀)(F,OH)₂ have also been used as sources of Fe(II) (Miot et al., 2009a; Shelobolina et al., 2012; Zhao et al., 2013). Some microorganisms prefer Fe(II) in the form of sulfate, sulfite or sulfide, as sulfur cannot be excluded as further source for microbial growth (see 4.1.2.2). Nevertheless, a significant influence of the Fe(II) source on the autotrophic denitrification extent has not been observed, but further studies are needed in order to assess the different mineralogy of Fe(III) precipitates.

2.4.2.5 Chelating agents

Chelating agents, such as EDTA and NTA have been used in several studies (Shelobolina et al., 2003; Kumaraswamy et al., 2006; Chakraborty et al., 2011; Carlson et al., 2013; Chakraborty and Picardal, 2013a; 2013b; Kanaparthi et al., 2013; Kanaparthi and Conrad, 2015; Kluglein et al., 2015). Fe-EDTA optimizes the process as the abiotic reactions

are minimized (Chakraborty and Picardal, 2013a) and prevents Fe(III) hydroxide mineral formation allowing the activity of microbes (Shelobolina et al., 2003; Chakraborty et al., 2011).

A complete Fe(II) oxidation was observed in 14 h by adding NTA and using *Desulfitobacterium frappieri* strain G2 as microbial culture (Shelobolina et al., 2003). However, in another experience the addition of EDTA resulted in a slower Fe(II) oxidation (5 d) than that obtained with aqueous Fe(II) (3 d) by *Dechloromonas* sp. UWNR4 (Chakraborty and Picardal, 2013b). Microbial activity is significantly influenced by the molar EDTA/Fe(II) ratio as microbial cultures differently deal with the toxic effects of Fe-EDTA species (Klueglein et al., 2015). Experiments with *Acidovorax* strains 2AN and BoFeN1 with a molar EDTA/Fe(II) ratio of 2:1 resulted in 71 and 47% Fe(II) oxidation in 25 d, respectively (Klueglein et al., 2015). On the contrary, when non-chelated Fe(II) was supplemented, Fe(II) oxidation reached 90 and 100% within 7 d by *Acidovorax* strains 2AN and BoFeN1, respectively, indicating the toxic effects of EDTA (Chakraborty et al., 2011). A molar EDTA/Fe(II) ratio of 1:1 did not promote the growth of an *Actinobacteria* culture and *T. denitrificans* (Kanaparthi et al., 2013; Kanaparthi and Conrad, 2015). However, the addition of 600 mg/L of humic substances as chelating agents resulted in 90 and 45% of Fe(II) oxidation and nitrate removal, respectively, by *T. denitrificans* (Kanaparthi and Conrad, 2015). In contrast, humic acids complexation with Fe(II) did not enhance Fe(II) bioavailability in experiments with *Acidovorax* sp. BoFeN1 (Larese-Casanova et al., 2010). A higher $[\text{Fe(II)EDTA}]^{2-}$ concentration was used by Kumaraswamy et al. (2006), and no inhibition on *P. ferrooxidans* strain BDN-1 was observed as Fe(II) oxidation occurred in 2 d.

2.4.2.6 Presence of organics

As many Fe(II) oxidizing denitrifiers are capable of growing under mixotrophic conditions, the supplementation of simple organic electron donors enhances Fe(II) oxidation and nitrate removal. Acetate is the most effective organic compound. Typically 1 mol of acetate is added per 2.5-10 moles of iron, depending on the microbial culture used (Straub et al., 1996; Benz et al., 1998; Kappler et al., 2005; Muehe et al., 2009; Chakraborty et al., 2011; Chakraborty and Picardal, 2013a).

Lactate and benzoate can also be used for Fe(II)-driven autotrophic denitrification as demonstrated in sediments incubation (Caldwell et al., 1999). A complete Fe(II) oxidation and 87% of nitrate removal occurred in 12 d in presence of lactate or benzoate, whereas an

inhibition of the process was reported without addition of organics. Pyruvate stimulates the activity of *Sphaerotilus natans* strain DSM 6575^T leading to a complete Fe(II) oxidation and approximately 90% of nitrate removal in 10 d of incubation (Park et al., 2014). Mixotrophic growth with 5.0 mM of glucose and 0.3 mM of Fe(II) resulted in a complete nitrate removal after 4 d by the strain *Klebsiella pneumoniae* L17 (Liu et al., 2014).

2.4.3 Continuous-Flow Experiments

Iron bioprecipitation and biorecovery as well as heterotrophic and autotrophic denitrification have been widely studied as single processes in continuous bioreactors (Das et al., 2005; Martins et al., 2011; Chowdhury and Ojumu, 2014; Lin et al., 2015; Montalvo et al., 2016). However, to date, only a few research has been carried out on the investigation of Fe(II)-mediated autotrophic denitrification in continuous experiments. Zhang et al. (2015) used an up-flow anaerobic sludge bed reactor (UASB) with a total working volume of 0.8 L. The reactor was inoculated with anaerobic granular sludge taken from a full-scale reactor treating paper mill effluents. The hydraulic retention time (HRT) was approximately 18 h in the first 22 d, and nitrate removal and Fe(II) oxidation were above 95%. Between days 22 and 32, HRT was slightly reduced to 17 h, resulting in 60 and 38% of nitrate removal and Fe(II) oxidation, respectively. Finally, the process was observed at its steady state during 32-47 d with nitrate and Fe(II) removals of 45 and 44%, respectively. The efficiency of the process decreased during the experimentation due to the decrease of HRT and pH. The percentage of nitrogen removal was above 95% as long as the effluent pH value was over 6.0 before dropping to 45% when pH decreased to 4.5. The bioaugmentation of the reactor with heterotrophic denitrifying cultures significantly enhanced the performance, as Fe(II) oxidation and NO₃⁻ removal reached 99 and 83%, respectively, in 15 d (Wang et al., 2015).

Fe(II) and As(III) oxidation coupled to autotrophic denitrification was investigated in two 420 mL continuous-flow sand packed bed columns, inoculated with activated sludge and operated at controlled 30°C with an HRT of 24 h (Sun et al., 2009b). Acetate was used as an additional electron donor in order to support microbial depletion of initial DO. One reactor was operated with nitrate as electron acceptor and showed up to 10-fold higher Fe(II) and As(III) removal than the reactor operated in absence of nitrate. During a 250 d operation, feed arsenic was reduced from approximately 570 to 10 µg/L. The iron precipitates contained 7 to 8-fold higher quantities of Fe and As than those observed in the reactor without nitrate,

indicating the importance of biological denitrification in Fe(II) oxidation and As immobilization through co-precipitation.

2.4.4 Influence of heavy metals and metalloids

Heavy metals such as iron, copper, cobalt and nickel can influence the activity of denitrifiers by stimulating or inhibiting the microbial activity (Zou et al., 2014). Generally, at higher concentrations metals alter the microbial enzyme conformation and block essential functional groups (Boopathy, 2000; Giller et al., 2009). On the contrary, if supplemented as trace elements, metals can enhance metabolic degradation (Gikas, 2007). Furthermore, longer exposure of the bacteria to heavy metals can increase their metal tolerance (Zou et al. 2015) through different mechanisms such as the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Spain, 2003).

Many studies reported the effect of heavy metals on heterotrophic denitrifying activity but metal impact on Fe(II) oxidizing denitrifiers has been poorly investigated. Only the effect of As, as both extra electron donor and inhibiting metal, has been studied. Enriched cultures of *Acidovorax* strain BoFeN1 and *Rhodobacter ferrooxidans* strain SW2 and the enrichment culture KS tolerated As(III) concentrations up to 20-50 μ M resulting in complete Fe(II) oxidation in 4, 14 and 8 d, respectively (Hohmann et al., 2009). The formation of biogenic Fe(III) minerals have the potential to co-precipitate As, mostly as As(V), affecting arsenic mobility and bioavailability towards the bacteria (Senn and Hemond, 2002; Sun, 2009a; Hohmann et al., 2009; Ahoranta et al., 2016). Similarly, Sun et al. (2009a) demonstrated that microbial oxidation of As(III) and Fe(II) linked to denitrification resulted in an enhanced immobilization of As(V) onto biogenic Fe(III) (hydr)oxides in sand packed bed columns.

As(III) is also a potential electron donor for chemolithotrophic denitrification. Li et al. (2015) studied the potential for simultaneous removal of nitrate, ferrous iron and arsenite from water. The pure culture of *Citrobacter freundii* strain PXL1 was enriched on arsenite concentration ranging between 2.85 and 13.65 mM. Almost complete Fe(II) oxidation occurred in 6 d, with 29% of As(III) removal while reducing 44% of nitrate.

2.4.5 Biogenic Fe(III) (hydr)oxide products by nitrate-dependent Fe(II) oxidation

Formation of biogenic Fe(III) (oxyhydr)oxides minerals from soluble Fe(II) and Fe(II)-containing mineral phases can be performed by different microorganisms (Posth et al., 2014). Besides the particular microbial culture used, different Fe(III) minerals are formed when changing the medium composition, the concentration of possible co-substrates and the incubation conditions (pH and temperature). The mineralogy of the formed Fe(III) minerals can be influenced and even controlled by the mechanism of Fe(II) oxidation, metabolic rates and presence of nucleation sites (Kappler and Straub, 2005; Senko et al., 2005a; Larese-Casanova et al., 2010; Pankte et al., 2012).

Different biogenic Fe(III) (hydro)oxide mineral phases are 2-line ferrihydrite, goethite, lepidocrocite, hematite, magnetite, maghemite and mixed-phase Fe(II)-Fe(III) minerals (green rust), (Weber, 2006a; Konhauser, 2011; Senko et al., 2005a;b). Table 2.3 shows the biogenic Fe(III)(hydr)oxide products deriving from Fe(II)-mediated autotrophic and mixotrophic denitrification.

Biogenic Fe oxides, such as lepidocrocite (γ -FeOOH) and goethite (α -FeOOH), have larger specific surface areas and higher binding energy than chemically produced Fe oxides, being thus more efficient for heavy metal adsorption (Hennebel et al., 2009). The surface of many Fe(III) minerals is positively charged at neutral pH and can adsorb negatively charged compounds such as arsenate, phosphate and organic matter (Kappler and Straub, 2005).

A rapid Fe(II) oxidation leads to the formation of ferrihydrite, while slower oxidation can result in green rust and other more crystalline minerals, such as goethite (Lack et al., 2002b; Senko et al., 2005b). Addition of FeCO₃ leads to the formation of Fe(III) oxyhydroxide or ferrihydrite, while the use of FeS results in the production of green rust (Hafenbradl et al., 1996). pH also rules the formation of Fe(III) minerals. In the experiments performed with *Acidovorax* sp. BoFeN1, lepidocrocite was detected at pH 6.3, while at pH 7.7 goethite was formed (Larese-Casanova et al., 2010).

Iron minerals are reported to be encrusted or non-encrusted, as the produced Fe(III) compound can form a crust around the cells during autotrophic or mixotrophic growth (Kappler et al., 2005; Posth et al., 2014). When Fe(II) oxidation is performed out of the cells, bacterial metabolism is not affected by encrustation (Benz et al., 1998; Schaedler et al., 2009; Ilbert and Bonnefoy, 2013; Posth et al., 2014). The presence of extracellular organic compounds that scavenge Fe(III) (Muehe et al., 2009) and the supplementation of chelating agents (i.e. EDTA)(Chakraborty and Picardal, 2013b) also prevent Fe(III) mineral

precipitation on the cell surface and encrustation. Chakraborty et al, (2011) reported that high feed Fe(II) and NO_3^- concentrations results in encrustation of the cells.

The color is also an indicator of the particular Fe(III) mineral formed. Mixed Fe(II)-Fe(III) hydroxides as major products cause a grayish-green appearance known as green rust (Straub et al., 1996). At a higher extent of Fe(II) oxidation to Fe(III), the precipitates turn into orange (Miot et al., 2009a). Generally, Fe(III) precipitates initially show a white fluffy structure that turns into a greenish-gray substance (carbonate-containing green rusts) within a week after the start of incubation (Chaudhuri et al., 2001). The white precipitates are identified as vivianite deriving from chemical Fe(II) oxidation in both biotic and abiotic experiments (Miot et al., 2009a). In biotic tests, after the formation of green rusts, Fe(III) precipitates become progressively orange and mainly consist of amorphous Fe(III)-phosphate (Miot et al., 2009a; Pantke et al., 2012). Also Fe(III) oxyhydroxide or ferrihydrite generally gives the liquid phase an orange/brown color (Lack et al., 2002b).

The potential for the reuse of sorbed and coprecipitated elements is an important aspect (Finneran, 2002). Green rust is a highly reactive iron mineral and potentially plays a key role for the fate of organic and inorganic contaminants, such as chromium or arsenic (Pantke et al., 2012). Fe(III) accumulated in the sludge of wastewater treatment plants can be recycled and used for phosphorus removal (Nielsen and Nielsen, 1998; Zhang et al., 2015). Efficient immobilization of arsenic by biogenic crystalline goethite was reported in Hohmann et al. (2009). Fe(III) (hydr)oxides can be also (re)used in different applications, such as pigments, catalysts, and co-precipitated with heavy metals (Cornell and Schwertmann, 2003).

Table 2.3: Biogenic Fe(III)(hydr)oxides produced during Fe(II)-mediated autotrophic (or mixotrophic) denitrification.

Species	Fe ²⁺ source	Inorganic carbon source, pH buffer	Biogenic Fe(III) minerals and end products	Details	References
<i>Azospira suillum</i> strain PS (formerly <i>Dechlorosoma suillum</i> strain PS)	FeCl ₂ (sol.)	HCO ₃ ⁻	Green rusts by chemical reaction with nitrate to magnetite	Rapid chemical Fe(II) oxidation of Fe(II) leads to ferrihydrite, while a slower kinetic results in green rust production	Chaudhuri et al., 2001
			Ferrihydrite		Lack et al., 2002a
	FeCl ₂ (sol.)	HCO ₃ ⁻	Crystalline Fe(III) mineral goethite	Poorly crystalline iron mineral phases are present, Fe(III) mineral crusts around cells	Kappler et al., 2005
	FeCl ₂ (sol.)	HCO ₃ ⁻	Fe(III) (hydr)oxides or Fe(III) phosphates	Fe(III) mineral crusts around the cells	Schaedler et al., 2009
<i>Acidovorax</i> strain BoFeN1	FeCl ₂ (sol.), vivianite (Fe ^{II} ₃ (PO ₄) ₂ , 8H ₂ O)	KH ₂ PO ₄	Vivianite and soluble Fe(II) lead to amorphous Fe(III)-phosphate	Dissolved Fe(II) is directly oxidized by the cells, while Fe(II) in vivianite is oxidized by nitrite produced by BoFeN1	Miot et al., 2009a
	FeCl ₂ (sol.)	KH ₂ PO ₄	Amorphous Fe(III)-phosphate	Precipitates are formed: 1) out of the cells in the bulk liquid, 2) on the cell surface, 3) in a mineralized layer within periplasm leading to cell encrustation	Miot et al., 2009b
	FeCl ₂ (sol.)	KH ₂ PO ₄	1 st phase: Goethite 2 nd phase: Nanogoethite, ferrihydrite, or lepidocrocite	Efficient immobilization of arsenic by production of biogenic goethite	Hohmann et al., 2009
	Acidic solution of ⁵⁷ Fe(II) by dissolving ⁵⁷ Fe(0)	MOPS, MES, NaCl, NaHCO ₃ , Na ₂ HPO ₄ , NaOH, NaCH ₃ COO	Lepidocrocite and goethite (Goethite produced by abiotic Fe(II) oxidation in the presence of high concentrations of HCO ₃ ⁻)	Neutral pH: production of lepidocrocite, Higher pH: phosphate or carbonate concentration and humic acids lead to the production of goethite	Larese-Casanova et al., 2010

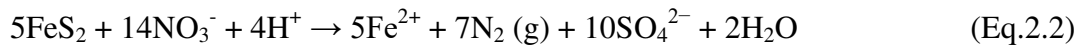
	FeCl ₂ (sol.)	HCO ₃ ⁻	Green rust transformed to goethite by chemical Fe(II) oxidation	Presence of extracellular Fe(III), Presence of high concentrations of bicarbonate lead to goethite	Pankte et al., 2012
	FeCl ₂ (sol.)	HCO ₃ ⁻ , low-phosphate mineral medium	Green rust transformed to goethite by chemical Fe(II) oxidation	Intermediate green-rust-like minerals oxidized by nitrite lead to goethite	Klueglein and Kappler, 2013
	FeCl ₂ (sol.)	Medium without phosphate, KH ₂ PO ₄	Green rust → Fe(III)-oxyhydroxides (lepidocrocite, goethite) → magnetite	Formation of stable single domain magnetite by bacteria	Miot et al., 2014
<i>Pseudogulbenkiania ferrooxidans</i> sp. strain 2002	Nontronite (NAu-2), Total Fe (24%), Fe(II) (0.6%)	HCO ₃ ⁻	Nontronite structure, but secondary minerals such as vivianite, ferrihydrite, and magnetite		Zhao et al., 2013
Enrichment culture and isolates (HidR2, BrG1, BrG2)	FeSO ₄	HCO ₃ ⁻	Ferrihydrite		Straub et al., 1996; 2004
Fe(II)-oxidizing enrichment culture KS	FeCl ₂ (sol.)	HCO ₃ ⁻	—	Non-encrustation in cells	Schaedler et al., 2009
	Structural Fe(II) in biotite	NaHCO ₃	Solid-state mineral transformation → Biotite alterations similar to those found in nature	Structural Fe(II) oxidation by direct enzymatic oxidation, followed by solid-state mineral transformation	Shelobolina et al., 2003
<i>Ferroglobus placidus</i>	FeCO ₃ , FeS	NaHCO ₃	FeCO ₃ → ferric oxyhydroxide or ferrihydrite and FeS → green rust	Observation in the color: ferric oxyhydroxide or ferrihydrite → rust brown, green rust → green gray	Hafenbradl et al., 1996
<i>Acidovorax</i> strain 2AN	FeCl ₂ , chelated Fe(II) (Fe(II)-EDTA), solid-phase Fe(II)	PIPES	Fe(III) oxyhydroxides	Use of chelators prevents cell encrustation	Chakraborty et al., 2011
<i>Sphaerotilus natans</i> strain DSM 6575 ^T	FeCl ₂	HCO ₃ ⁻	Formation of amorphous or less crystalline Fe(III) oxide minerals [Abiotic reaction → goethite]	Nanoball-shaped amorphous Fe(III) oxide minerals encrusting the cell surfaces	Park et al., 2014

<i>Citrobacter freundii</i> strain PXL1	FeCl ₂	Potassium sodium tartrate (C ₄ H ₄ O ₆ KNa·4H ₂ O)	Poorly crystalline Fe(III) oxides	Amorphous iron oxides mainly consisted of Fe (28.57%), O (24.59%), and K (23.69%) by weight.	Li et al., 2014; 2015
Anaerobic, nitrate- dependent enrichment culture obtained from a landfill leachate impacted aquifer	FeS	HCO ₃ ⁻	Lepidocrocite [Fe(III)] produced by oxidation of Fe(II) by nitrite was an amorphous hydrous ferric oxide (HFO)		Senko et al., 2005a
	FeCl ₂	Hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES)	Goethite		
<i>Klebsiella</i> Strain FW33AN	FeCl ₂		Goethite, (Crystalline Fe(III) (hydr)oxides)	Rapid oxidation rate → amorphous Fe(III)(hydr)oxide Slower oxidation → more crystalline goethite	Senko et al., 2005b
		HCO ₃ ⁻		Larger proportion of Fe(III)→crystalline fraction Phosphate→ controlling Fe(III) crystallinity	
	FeS		Amorphous Fe(III) (hydr)oxide		
Anaerobic sludge from lab-scale As(III)-oxidizing denitrifying bioreactor	FeCl ₂	HCO ₃ ⁻	Mixture of Fe(III) oxides dominated by crystalline hematite	Formation of Fe(III) oxides due to nitrate- dependent oxidation of soluble Fe(II)	Sun et al., 2009a

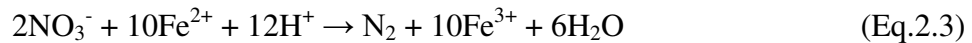
2.4.6 Supplementation of Fe(II) in form of pyrite (FeS₂)

Pyrite (FeS₂) is the most abundant sulfide mineral on the earth's crust and a major source for both iron and sulfur, playing an important role in iron and sulfur cycles (Bosch et al., 2012). Under aerobic conditions, iron and sulfur can be oxidized by aerobic chemolithotrophic microorganisms such as *Acidithiobacillus ferrooxidans* as it usually occurs in bioleaching applications. However, pyrite oxidation is not limited to aerobic environments because anaerobic pyrite oxidation is thermodynamically feasible involving nitrate as an electron acceptor (Bosch et al., 2012).

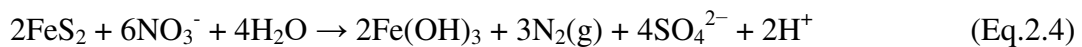
Pyrite can be a promising electron donor for denitrification especially in mining nitrate-environments where pyrite is abundant. The reaction between pyrite and nitrate is proton-consuming, with Fe(II), sulfate, and nitrogen gas as end products (Baeseman et al., 2006):



Fe(II) is subsequently oxidized:



Resulting in the following overall reaction:



Pyrite-driven autotrophic denitrification depends on several factors i.e. pyrite grain size, initial nitrate concentration, nitrate-loading rate and pH (Torrento et al., 2011). Pyrite is stable under severely reducing conditions or at pH lower than 7.4 whereas it is oxidized at higher pH.

Several studies reported the occurrence of nitrate reduction coupled to pyrite oxidation (Schwientek et al., 2008; Jorgensen et al., 2009; Zhang et al., 2009; Bosch et al., 2012; Zhang et al., 2012), indicating that pyrite is an effective electron donor capable of stimulating denitrification even in presence of organics (Zhang et al., 2012). However, in comparison with other compounds, lower denitrification efficiencies have been observed when using pyrite as electron donor. Bosch et al. (2012) observed a 10% pyrite oxidation to Fe(III) with a nitrate removal efficiency of 15% by using *T. denitrificans*. The addition of pyrite increased the proportion of denitrifying bacteria closely related to the *Xanthomonadaceae* species, responsible for autotrophic denitrification (Torrento et al., 2011).

The main shortcomings of pyrite oxidation coupled to denitrification are sulfate production and trace metal release (e.g. nickel and arsenic) as well as the production of additional intermediate products such as nitrite and nitrous oxide (Zhang et al., 2012).

2.5 Conclusions

Fe(II)-mediated autotrophic denitrification is a promising biotechnology for concomitant nitrate and ferrous iron removal and recovery/reuse of iron in the form of Fe(III) minerals. Many microorganisms are capable of performing the process as pure cultures or in mixed incubations. The use of neutral or slightly alkaline pH is encouraged as most microbial species are neutrophilic and Fe(II)-driven denitrification results in the production of acidity. Pyrite can be a promising electron donor for Fe(II) mediated autotrophic denitrification, as it is abundant in mining environments. Feed Fe(II) concentrations as high as 25 mM are generally tolerated by microorganisms, but the use of EDTA as Fe chelator at neutral pH can result in an inhibition of the microbial activity. In most cases, nitrate removal can be enhanced by supplementing small amounts of acetate or other simple organics. The biogenic Fe(III) minerals produced have potential to be reused in environmental and commercial applications. However, the mineral characterization of the biogenic Fe(III) precipitates has been poorly investigated. Additional work is also required to better investigate the influence of other metals on Fe(II)-mediated denitrification and the potential of the process in continuous-flow experiments for a possible future application in full-scale plants.

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CHAPTER 3

Influence of pH, EDTA:Fe(II) ratio and microbial culture on Fe(II)-mediated autotrophic denitrification

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3.1 Introduction

Nitrate is one of the most common pollutants in water resources worldwide (Park and Yoo, 2009; Zhang *et al.*, 2015). Nitrate contamination is mainly caused by the use of agricultural fertilizers (Viers *et al.*, 2012; Qambrani *et al.*, 2013) and the uncontrolled discharge of industrial and domestic wastewaters (Zhang *et al.*, 2015; Bhandari *et al.*, 2016). Moreover, nitrate is often associated with mining activity due to use of large amounts of explosives such as ‘ammonium nitrate fuel oil’ (ANFO) (Zaitsev *et al.*, 2008). In mining environments, nitrate co-occurs with several metal species, including iron (Papirio *et al.*, 2014).

Nitrate is mainly removed from wastewaters by heterotrophic denitrification (Ashok and Hait, 2015). Denitrification can also be used for the treatment of metal-containing wastewaters (Zou *et al.*, 2014, 2015). Fe(II) has been demonstrated to stimulate heterotrophic denitrification (Papirio *et al.*, 2014) and is also an effective electron donor for autotrophic denitrification (Straub *et al.*, 1996). The first microorganisms capable of maintaining biological nitrate-dependent Fe(II) oxidation were discovered only 20 years ago (Straub *et al.*, 1996). The use of denitrifying Fe(II) oxidizers results in the reduction of nitrate to nitrogen gas and the bioprecipitation/biorecovery of Fe(III) (hydr)oxides, with the possible co-precipitation or adsorption of other metals (Hohmann *et al.*, 2009; Ahoranta *et al.*, 2016). The complete reduction of nitrate to dinitrogen gas with Fe(II) as electron donor is as suggested by Sorensen (1987) (see eq.2.1).

Fe(II)-mediated autotrophic denitrification is advantageous over classical heterotrophic denitrification for the treatment of low-organic wastewaters. The addition of simple organic compounds would increase the operational costs and induce secondary organic pollution (Zhang *et al.*, 2015). Nevertheless, the use of waste activated sludge fermentation liquid as carbon source can enhance the denitrification efficiency, by keeping the treatment costs low as no organic substrates are supplemented (Ji and Chen, 2010). Moreover, the anaerobic methane oxidation coupled to denitrification can be considered as an alternative for the treatment of organic-deficient wastewaters (Wang *et al.*, 2017).

Nitrate dependent Fe(II) oxidation has been reported in both mixed enrichments and pure cultures isolated from various habitats (Weber *et al.*, 2006a; Kiskira *et al.*, 2017). A circumneutral feed pH and a molar Fe(II)/NO₃⁻ ratio of 5 have mostly been used in the existing literature (Straub *et al.*, 1996; Weber *et al.*, 2006b; Blöthe and Roden, 2009). None

of the known Fe(II)-oxidizing denitrifiers has been reported to be acidophilic, and the knowledge of their possible acclimation to acidic environments still remains limited.

Thiobacillus denitrificans and *Pseudogulbenkiania* strain 2002 are microorganisms capable of performing Fe(II)-driven autotrophic denitrification. The two microbial species are both located in the subclass of the *Proteobacteria* and are reported to grow as anaerobic chemolithotrophs (Kelly and Wood, 2000; Weber *et al.*, 2006b). *T. denitrificans* can be found in soil, mud, freshwater, marine sediments and also in domestic sewage and industrial wastewater treatment ponds. It has widely been reported to use reduced sulfur compounds (e.g. thiosulfate, elemental sulfur and sulfide) as electron donors, but contradictory results have been obtained for the capability of *T. denitrificans* to use Fe(II) (Straub *et al.*, 1996; Muehe *et al.*, 2009). *Pseudogulbenkiania* strain 2002 has been observed in freshwater and paddy soils used for the cultivation of rice and soybean (Tago *et al.*, 2011). Unlike *T. denitrificans*, *Pseudogulbenkiania* strain 2002 is a strictly nitrate-dependent Fe(II) oxidizing microorganism (Weber *et al.*, 2006b, 2009).

As Fe(II) is not stable at circumneutral pH, ethylenediaminetetraacetic acid (EDTA) is commonly employed as chelating agent in order to promote a higher Fe(II) solubilization. An attentive supplementation of EDTA is required, as EDTA is an organic pollutant which is persistent in the environment (Oviedo and Rodríguez, 2003). Moreover, EDTA often leads to the inhibition of the activity of several denitrifying Fe(II)-oxidizing species (Kumaraswamy *et al.*, 2006; Kanaparthi *et al.*, 2013; Klueglein *et al.*, 2015). Further research is therefore needed in order to evaluate the most suitable EDTA:Fe(II) ratio that does not result in microbial inhibition, while enhancing Fe(II) solubility. For instance, EDTA can be recycled and reused, mitigating the organic pollution and lowering the operational costs (Juang and Wang, 2000).

The objectives of this work were 1) to investigate the efficiency of Fe(II)-mediated autotrophic denitrification in terms of Fe(II) oxidation and nitrate removal with different microbial cultures in batch bioassays; 2) to evaluate the effects of decreasing pH on the process; 3) to determine the optimal EDTA/Fe(II) ratio.

3.2 Materials and Methods

3.2.1 Sources of microorganisms and cultivation mineral media

The chemolithotrophic denitrifying cultures used in this study were: (1) a *Thiobacillus*-dominated mixed culture previously enriched on thiosulfate and nitrate (Di Capua *et al.*, 2016; Zou *et al.*, 2016); (2) an activated sludge inoculum collected from the municipal wastewater plant in Cassino (Italy); (3) a pure culture of *Pseudogulbenkiania* strain 2002 (DSM 18807); (4) a pure culture of *T. denitrificans* (DSM 12475). Both pure cultures were purchased from the ‘Leibniz-Institute DSMZ- German collection of microorganisms and cell cultures’ in Braunschweig (Germany).

The two denitrifying mixed cultures were enriched under anaerobic conditions for 1 month in batch mode in 125 mL serum flasks. Fe(II) and NO_3^- concentrations were 10 and 2 mM, respectively. The basal medium was prepared with the following components ($\text{g}\cdot\text{L}^{-1}$): 2.00 NaHCO_3 , 0.25 NH_4Cl , 0.30 KH_2PO_4 , 0.40 $\text{K}_2\text{H}_2\text{PO}_4$, and 0.10 NaCl . The trace mineral solution was added from a sterile stock solution and prepared by dissolving the following in a 1.5 g L^{-1} nitrilotriacetic acid disodium salt solution ($\text{g}\cdot\text{L}^{-1}$): 3.00 $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.50 MnSO_4 , 1.00 NaCl , 0.10 $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.10 $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.10 $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 0.13 ZnCl , 0.01 $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 0.01 $\text{AlK}(\text{SO}_4)_2\cdot 12\text{H}_2\text{O}$, 0.01 H_3BO_3 , 0.025 $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ (Weber *et al.*, 2009).

The two pure cultures were preliminary cultivated by using two different mineral media. The mineral medium used for the *Pseudogulbenkiania* strain 2002 culture contained the following components ($\text{g}\cdot\text{L}^{-1}$): 5.0 peptone, 2.0 meat extract, and 15.0 agar. pH was adjusted to 7.0. The medium was sterilized by autoclaving at 121°C for 15 min. The medium used for the activation of the *T. denitrificans* culture consisted of four different solutions, as reported by Zou *et al.* (2016). Fe(II) and NO_3^- concentrations were the same of those used for the cultivation of *Thiobacillus*-mixed and activated sludge cultures.

All the incubations were maintained in absence of light at $22 \pm 2^\circ\text{C}$ on a gyratory shaker (80 rpm). After the enrichment, all the microbial cultures were seeded in the serum bottles used for the batch experiments.

3.2.2 Preparation of the experiments

Fe(II)-mediated autotrophic denitrification was studied in batch bioassays by using 125 mL serum bottles. All the bottles were maintained at room temperature ($22 \pm 2^\circ\text{C}$). Each bottle contained the basal medium and trace elements, as described in section 2.1. Fe(II) and NO_3^- were added in concentration of 10 and 2 mM, respectively. Thiosulfate ($\text{S}_2\text{O}_3^{2-}$) in concentration of 0.5 mM was used as an additional electron donor in the experiments performed with the *Thiobacillus*-mixed and pure *T. denitrificans* cultures. Fe(II), NO_3^- and $\text{S}_2\text{O}_3^{2-}$ were added in the form of iron(II) chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), sodium nitrate (NaNO_3) and sodium thiosulfate (NaS_2O_3), respectively. EDTA in molar ratios of 2:1, 1:1 and 0.5:1 with Fe(II) was used as chelating agent. All the chemicals were of analytical grade (Sigma Aldrich, Germany).

The feed pH (7.0, 6.0 and 5.0) was adjusted by adding NaOH and HCl before flushing the bottles with He in order to maintain anoxic conditions. The dissolved oxygen (DO) was below $0.3 \text{ mg} \cdot \text{L}^{-1}$. Bicarbonate ($2 \text{ g} \cdot \text{L}^{-1}$ as NaHCO_3) was added to each bottle as buffer and inorganic carbon source. The enrichment cultures were added to the bottles in the amount of 10% v/v. This resulted in an initial volatile suspended solids (VSS) concentration of 180, 300, 380 and $720 \text{ mg VSS} \cdot \text{L}^{-1}$ in the bottles inoculated with *Thiobacillus*-mixed, pure *T. denitrificans*, activated sludge and *Pseudogulbenkiania* strain 2002 cultures, respectively. Finally, the bottles were sealed with butyl rubber stoppers and aluminum crimps and placed on a gyratory shaker at 250 rpm. Microcosms were prepared in duplicate. For each microbial culture, controls without electron donors were carried out to monitor the degradation of NO_3^- , which was not associated with chemolithotrophic denitrification. Abiotic controls were also performed for possible chemical reactions between Fe(II), NO_3^- and/or $\text{S}_2\text{O}_3^{2-}$.

3.2.3 Batch bioassays

The batch experiments were conducted as described in Table 3.1. The four inocula were individually investigated in each experiment. In Experiment 1, Fe(II) was used as sole electron donor for chemolithotrophic denitrification using all the four cultures at pH 7.0 and a EDTA:Fe(II) ratio of 2.0. In Experiment 2, the potential of thiosulfate as additional electron donor was investigated in the bioassays seeded with the *Thiobacillus*-mixed and pure *T. denitrificans* cultures under the same operating conditions of Experiment 1. Thiosulfate was

added in concentration of 0.5 mM, lower than the theoretical amount indicated by the molar $\text{NO}_3^-:\text{S}_2\text{O}_3^{2-}$ ratio of 1.6:1.0 (Manconi *et al.*, 2007).

In Experiment 3, the *Thiobacillus*-mixed and pure *T. denitrificans* cultures cultivated on Fe(II) and thiosulfate and the activated sludge and *Pseudogulbenkiania* strain 2002 cultures cultivated on the sole Fe(II) were subcultured in a new medium prepared at pH 7.0 and with a EDTA:Fe(II) ratio of 2.0. In Experiment 4, the effect of decreasing EDTA concentrations (20, 10 and 5 mM) and pH (7.0 and 6.0) was evaluated by using the enrichment cultures from Experiment 3. In Experiment 5, the use of Fe(II) as sole electron donor at pH 5.0 and EDTA:Fe(II) ratio of 0.5 was investigated by using the enriched *Thiobacillus*-mixed, pure *T. denitrificans* and activated sludge cultures from Experiment 3.

Table 3.1: Operating conditions used in the batch experiments. Feed nitrate was 2 mM in all the bioassays.

Experiment	Microbial cultures	Electron donor	EDTA:Fe(II)	pH
1	<i>Thiobacillus</i> -dominated mixed culture (TM)	Fe(II) – 10 mM	2.0	7
	<i>T. denitrificans</i> pure culture (TDP)			
	Activated sludge (AS)			
	<i>Pseudogulbenkiania</i> strain 2002 (PG)			
2	TM (enriched on Fe(II) from Experiment 1)	Fe(II) – 10 mM $\text{S}_2\text{O}_3^{2-}$ - 0.5 mM	2.0	7
	TDP (acclimated to Fe(II) from Experiment 1)			
3	TM (enriched on $\text{S}_2\text{O}_3^{2-}$ and Fe(II) from Experiment 2)	Fe(II) – 10 mM	2.0	7
	TDP (acclimated to $\text{S}_2\text{O}_3^{2-}$ and Fe(II) from Experiment 2)			
	AS (enriched on Fe(II) from Experiment 1)			
	PG (acclimated to Fe(II) from Experiment 1)			
4	TM (from Experiment 3)	Fe(II) – 10 mM	2.0	7
	TDP (from Experiment 3)		1.0	6
	AS (from Experiment 3)		0.5	
	PG (from Experiment 3)			
5	TM (from Experiment 4)	Fe(II) – 10 mM	0:5	5
	TDP (from Experiment 4)			
	AS (from Experiment 4)			

3.2.4 Sampling and analytical methods

Ferrous iron, nitrate, thiosulfate and pH were analyzed at t=0, after 6 h on day 1 and, subsequently, every 24 h after day 1 until day 10. Samples were taken with 5-mL disposable syringes. NO_3^- and $\text{S}_2\text{O}_3^{2-}$ concentration was analyzed by ion chromatography (IC) with chemically suppressed conductivity using a 883 Basic IC Plus system equipped with a Metrosep A Supp 5-150/4.0 column and a 863 Compact IC Autosampler (Metrohm, Switzerland). The liquid samples were filtered with 0.22 μm syringe cellulose membranes

(EMD Millipore, USA) prior to IC analysis. Ferrous iron was quantified photometrically by using a Lambda 10 UV-Vis spectrometer (Perkin Elmer, USA), following the analytical method reported by Ahoranta *et al.*, (2016). Fe(II) determination was performed immediately after the sampling, for avoiding Fe(II) chemical oxidation. DO and pH measurements were performed with a Multimeter 3410 (WTW, Germany) equipped with a FDO® 925 and a SenTix® 140-3 pH electrode, respectively. VSS were analyzed according to the standard methods (APHA, 1992). Gas samples were not taken from the headspace of the bottles. The production of NO and N₂O was not evaluated.

3.3 Results and discussion

3.3.1 Fe(II)-mediated autotrophic denitrification with pH 7.0 and EDTA:Fe(II) 2.0

3.3.1.1 Supplementation of Fe(II) as sole electron donor with the four initial cultures

Fe(II)-mediated autotrophic denitrification was investigated in batch experiments under different operating conditions. Table 3.2 reports the nitrate removal and Fe(II) oxidation achieved in all the batch bioassays after 10 d. In all experiments, no nitrite was detected as intermediate of denitrification and pH remained stable at 7.0.

Fig. 3.1a shows the results obtained in Experiment 1, using Fe(II) as sole electron donor in the bioassays seeded with the four initial inocula. The pure *T. denitrificans* culture was capable of maintaining Fe(II)-mediated autotrophic denitrification. The specific average nitrate removal rate was 2.7 mg/(g VSS·d). The molar Fe(II):NO₃⁻ ratio constantly ranged between 3.5 and 5.0, indicating that denitrification proceeded in a good agreement with the stoichiometry (Eq. 3.1). After 10 d, Fe(II) oxidation reached 66%, whereas nitrate removal was 52%. Conversely, denitrification did not occur in the bioassays with the *Thiobacillus*-mixed culture. Fe(II) oxidation was 35%, but not associated with nitrate removal. Fe(II) was oxidized most likely due to the chemical reaction with residual DO, in agreement with what observed in abiotic controls. The profile of Fe(II) in the abiotic controls was as reported in Fig. 3.1a. Fe(II) concentration decreased by 35% in the first 4 d due to chemical oxidation. NO₃⁻ concentration did not significantly change over 10 d in both free electron donor and abiotic controls.

Table 3.2: NO₃⁻ removal and Fe(II) oxidation rates and efficiency achieved in all the bioassays

Experiment	Microbial culture	EDTA:Fe(II)	pH	Time [d]	NO ₃ ⁻ removal [%]	Specific NO ₃ ⁻ removal rate [mg/(g VSS·d)]	Fe(II) oxidation [%]	Specific Fe(II) oxidation rate [mg/(g VSS·d)]
1	TM	2.0	7	0-10	-	-	35	14.0
	TDP				52	2.7	66	12.5
	AS				72	2.4	83	9.5
	PG				55	1.0	54	4.6
2	TM	2.0	7	0-4	73	14.7	37	12.2
				5-10	9	1.2	44	9.7
				0-10	82	6.6	81	10.7
	TDP			0-5	49	4.7	43	16.5
				6-10	26	2.5	30	11.5
				0-10	75	3.6	73	14.0
3	TM	2.0	7	0-10	83	8.6	68	11.2
	TDP				82	4.4	95	17.7
	AS				65	2.6	45	5.1
	PG				54	1.2	64	4.7
4	TM	2.0	7	0-10	80	8.8	54	20.3
		1.01			76	10.3	74	29.5
		0.5			98	10.5	83	30.2
		2.0	6		85	10.4	65	23.6
		1.0			98	10.1	60	22.7
		0.5			98	10.5	56	26.5
	TDP	2.0	7		83	4.7	81	18.9
		1.0			78	6.1	70	15.5
		0.5			93	5.9	89	17.0
		2.0	6		47	2.4	78	15.5
		1.0			56	2.6	60	12.0
		0.5			66	3.3	65	12.8
	AS	2.0	7		91	4.3	64	10.5
		1.0			89	4.7	60	9.6
		0.5			100	4.8	89	12.0
		2.0	6		84	4.9	59	9.8
		1.0			100	5.1	82	14.5
		0.5			100	5.2	100	12.1
	PG	2.0	7		61	1.8	40	3.7
		1.0			96	2.5	57	5.1
		0.5			100	3.3	53	5.6
		2.0	6		64	1.7	43	3.9
		1.0			77	2.2	42	3.9
		0.5			89	2.5	29	2.0
5	TM	0.5	5	0-10	45	3.7	40	13.9
	TDP				30	1.5	32	7.0
	AS				39	1.5	49	7.8

The feasibility of Fe(II)-driven denitrification with a pure culture of *T. denitrificans* is still contradictory. Straub et al. (1996) observed nitrate-dependent Fe(II) oxidation under strictly autotrophic conditions with a Fe(II):NO₃⁻ molar ratio of 5:1. Fe(II) oxidation occurred in 8 weeks, but no information for nitrate removal was presented. In contrast, Muehe *et al.* (2009) did not observe Fe(II) oxidation coupled to autotrophic denitrification. The addition of organic compounds, i.e. humic substances, was needed to stimulate both Fe(II) and NO₃⁻ removal, likely due to the enhanced Fe(II) complexation and bioavailability (Kanaparthi and Conrad, 2015).

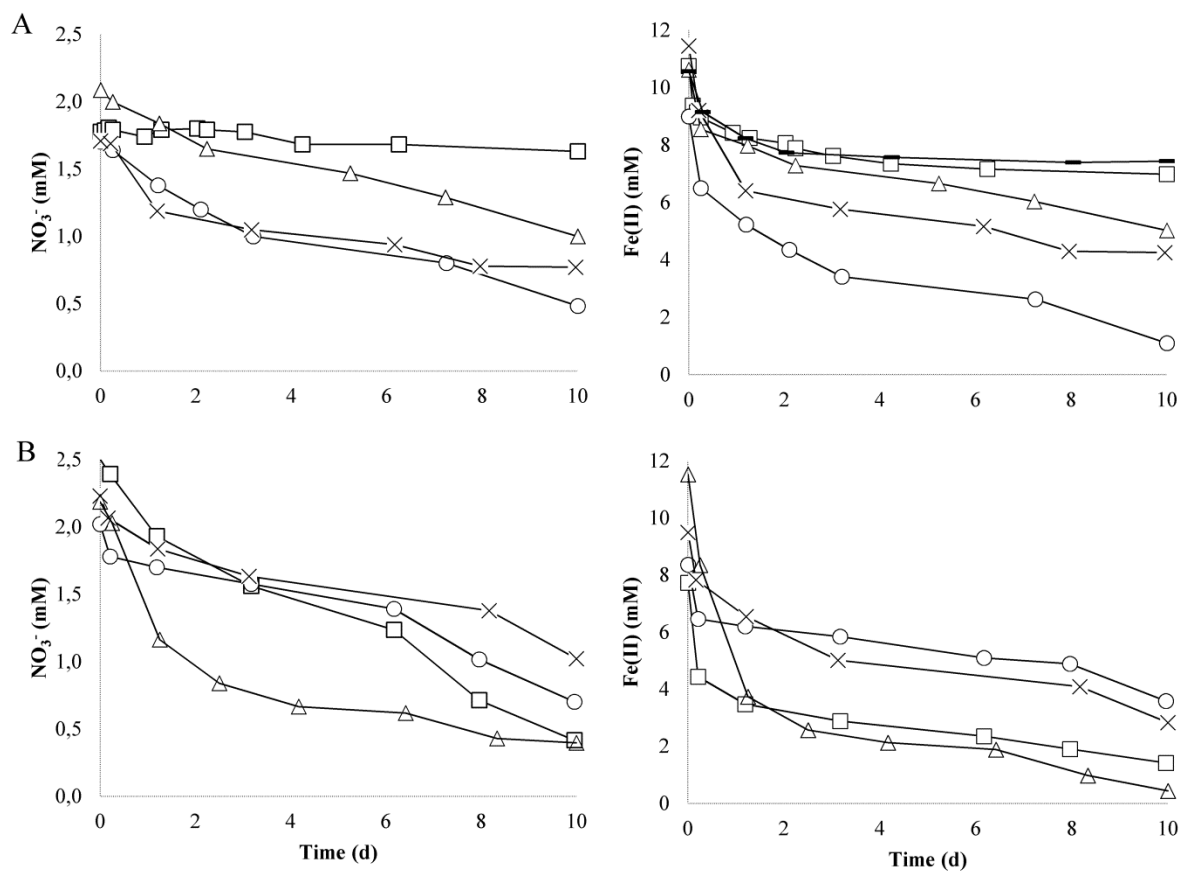


Figure 3.1: NO₃⁻ and Fe(II) profiles in Experiment 1 (A) and Experiment 3 (B) in abiotic controls (-) and bioassays with the *Thiobacillus*-mixed culture (TM) (□), pure *T. denitrificans* culture (TDP) (Δ), activated sludge inoculum (AS) (○) and *Pseudogulbenkiania* strain 2002 culture (PG) (x). Standard deviations are in the range 0.02-0.65 mM.

In this study, the specific nitrate removal rate obtained with activated sludge inoculum was 2.4 mg/(g VSS·d), slightly lower than that achieved with *T. denitrificans* (Fig. 3.1a). Fe(II) oxidation and nitrate removal were 83% and 72%, respectively, at the end of the experiments. The molar ratio between oxidized Fe(II) and removed nitrate was in the range 2.2 - 4.8, in agreement with Nielsen and Nielsen (1998). However, Nielsen and Nielsen

(1998) reported a much faster Fe(II)-based denitrification as 3 mM of Fe(II) was oxidized in 5 h coupled to the removal of 1 mM of nitrate.

Although *Pseudogulbenkiania* strain 2002 is a more specialized microbial culture in maintaining Fe(II)-based autotrophic denitrification, the use of *Pseudogulbenkiania* strain 2002 resulted in the lowest specific NO_3^- removal rate, i.e. 1.0 mg/(g VSS·d) (Fig. 3.1a). Fe(II) oxidation reached 54%, whereas nitrate removal was 55%. The molar Fe(II): NO_3^- ratio was in the range 4.6-5.5, in a good agreement with the stoichiometry (Eq. 2.1). The high EDTA concentration most probably repressed the microbial activity of *Pseudogulbenkiania* strain 2002. A slow nitrate removal with *Pseudogulbenkiania* strain 2002 was also observed by Weber *et al.* (2006b), even in the absence of EDTA. In their study, *Pseudogulbenkiania* strain 2002 only oxidized 25% of the initial 10 mM Fe(II) and 22% of the fed 2.2 mM nitrate in 7 d.

3.3.1.2 Supplementation of $\text{S}_2\text{O}_3^{2-}$ as additional electron donor to the *Thiobacillus*-mixed and pure *T. denitrificans* cultures

The capability for Fe(II)-mediated autotrophic denitrification of the previously Fe(II)-enriched *Thiobacillus*-mixed culture was investigated by supplementing thiosulfate in Experiment 2 (Fig. 3.2). Thiosulfate was completely oxidized in 4 d. At this stage, nitrate removal reached up to 73% with a specific nitrate removal rate of 14.7 mg/(g VSS·d). About 46% of the initial nitrate was removed by thiosulfate-driven denitrification, according to the stoichiometry reported by Manconi *et al.* (2007). The remaining 27% of nitrate was removed by Fe(II)-oxidizing metabolism. Aside the initial Fe(II) drop to 4 mM due to chemical oxidation, Fe(II) oxidation was 37% after 4 d, indicating that Fe(II) oxidation was almost completely associated with autotrophic denitrification. From day 5 on, nitrate removal was only 9% with Fe(II) as sole electron donor. As also observed in Experiment 1, the *Thiobacillus*-mixed culture was not able to maintain Fe(II)-mediated autotrophic denitrification in absence of thiosulfate. The specific nitrate removal rate dropped to 1.2 mg/(g VSS·d), whereas the iron oxidation rate remained constant.

The effect of thiosulfate as supplementary electron donor was also tested on the pure *T. denitrificans* culture (Fig. 3.2). As long as thiosulfate was present, the specific nitrate removal rate was 4.7 mg/(g VSS·d). In the absence of thiosulfate, denitrification proceeded

with a nitrate removal rate of 2.5 mg/(g VSS·d). The overall nitrate removal rate was 3.6 mg/(g VSS·d), i.e. 34% higher than that achieved in Experiment 1.

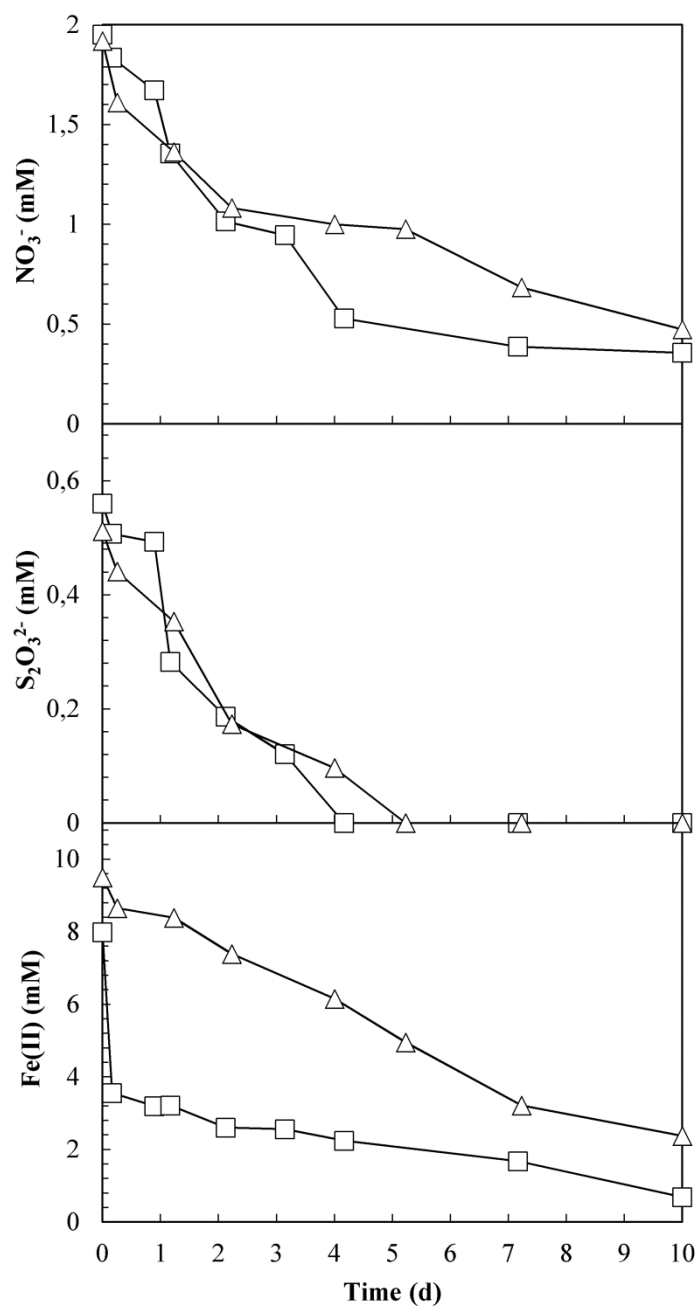


Figure 3.2: The evolution of NO_3^- , $\text{S}_2\text{O}_3^{2-}$ and Fe(II) during Experiment 2 for TM (□) and TDP (Δ). Standard deviations are in the range 0.02-0.65.

Both the *Thiobacillus*-mixed and pure *T. denitrificans* cultures were stimulated by supplementing thiosulfate as additional electron donor. However, the increase of nitrate removal rate was more significant for *Thiobacillus*-mixed culture, as the prolonged enrichment of the *Thiobacillus*-mixed culture on $\text{S}_2\text{O}_3^{2-}$ in a previous study (Di Capua *et al.*, 2016) resulted in a microbial community specialized in using thiosulfate as sole electron

donor. Conversely, the pure *T. denitrificans* culture demonstrated to use Fe(II) more efficiently than the *Thiobacillus*-mixed culture.

3.3.1.3 Supplementation of Fe(II) as sole electron donor with all the acclimated and enriched cultures

After enriching and acclimating the *Thiobacillus*-mixed and pure *T. denitrificans* cultures on thiosulfate, Fe(II)-based denitrification was again investigated with Fe(II) as sole electron donor. The use of a more enriched *Thiobacillus*-mixed culture resulted in a higher biological Fe(II) oxidation coupled to denitrification (Fig. 3.1b). The specific nitrate removal rate significantly increased and reached 8.6 mg/(g VSS·d). Fe(II) oxidation and NO₃⁻ reduction were 68 and 83%, respectively. *Thiobacillus thioparus* predominated over *T. denitrificans* in the microbial community (Di Capua *et al.*, 2016). *T. thioparus* has previously been reported as an obligate thiosulfate-oxidizing chemolithotrophic denitrifier, not capable of using Fe(II) (Robertson and Kuenen, 2006). However, after a prolonged enrichment on Fe(II) and S₂O₃²⁻, the combined Fe(II)-oxidizing activity of *T. thioparus* and *T. denitrificans* was considerably enhanced, also in the presence of Fe(II) as sole electron donor.

Denitrification coupled to Fe(II) oxidation was also stimulated by subculturing the pure *T. denitrificans* culture in a fresh medium. The specific nitrate removal rate was 4.4 mg/(g VSS·d), compared to 2.7 mg/(g VSS·d) obtained in Experiment 1. Nitrate removal was 82% and Fe(II) oxidation was 95%.

In contrast, nitrate removal in the bioassays with activated sludge and *Pseudogulbenkiania* strain 2002 cultures was less significantly enhanced after the subculture of the microbial cells (Fig. 3.1b). Compared to Experiment 1, the specific nitrate removal rate only increased by 8% for activated sludge inoculum, whereas it rose from 1.0 to 1.2 mg/(g VSS·d) for *Pseudogulbenkiania* strain 2002. Nitrate removal and Fe(II) oxidation were 66 and 45% for activated sludge inoculum and 54 and 64% for *Pseudogulbenkiania* strain 2002, respectively. A slower Fe(II) oxidation was observed for the activated sludge inoculum than that obtained in Experiment 1, most probably due to an optimization of denitrification that required a lower Fe(II) amount to achieve the same nitrate removal.

The rates of Fe(II)-mediated autotrophic denitrification obtained in this work were lower than those reported in similar batch experiments aimed at investigating classical heterotrophic denitrification or sulfur-driven autotrophic denitrification. For instance, Papirio

et al. (2014) observed a nitrate removal rate up to approximately $400 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ by using denitrifying cultures enriched on ethanol. A N-NO_3^- removal rate of $52.2 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, i.e. 3-fold higher than the highest achieved in this study, was obtained by Di Capua *et al.* (2016) under chemolithotrophic conditions with $\text{S}_2\text{O}_3^{2-}$ as electron donor. However, the implementation of Fe(II)-mediated autotrophic denitrification in continuous-flow bioreactors is expected to result in higher nitrate removal rates. Under these operating conditions, a higher biomass concentration can be used leading to an enhanced denitrification efficiency (Zhang *et al.*, 2015).

In comparison with heterotrophic and sulfur-based autotrophic denitrification, Fe(II)-mediated autotrophic denitrification does not result in nitrite and this represents a major advantage as nitrite is reported to be inhibitory for many denitrifiers and nitrogen can be entirely removed from the liquid phase (Straub *et al.*, 1996; Zhang *et al.*, 2015).

3.3.2 Effect of the EDTA:Fe(II) ratio

EDTA is widely used in many environmental applications such as metal recovery from wastewaters and soils (Di Palma *et al.*, 2003). The use of EDTA results in increasing treatment costs, but EDTA can be effectively recycled and suitable for reuse without losing its chelating properties (Juang and Wang, 2000; Di Palma *et al.*, 2003). In Fe(II)-mediated autotrophic denitrification, EDTA is used to improve Fe(II) solubility and bioavailability. An EDTA-recycling step after denitrification can be an interesting option to enhance the economic feasibility of the overall process (Zeng *et al.*, 2005).

The dosing of EDTA is of major importance in Fe(II)-based denitrification. Microbial activity can be influenced by the molar EDTA:Fe(II) ratio as microbial cultures differently tolerate the inhibitory effects of free EDTA and Fe-EDTA species (Klueglein *et al.*, 2015). In this study, the effect of decreasing EDTA on Fe(II)-mediated autotrophic denitrification was investigated in Experiment 4. The NO_3^- removal efficiency achieved at EDTA:Fe(II) ratios of 2.0, 1.0 and 0.5 was as shown in Fig. 3.3.

When using the *Thiobacillus*-mixed culture, the specific nitrate removal rate was 8.8, 10.3 and $10.5 \text{ mg}/(\text{g VSS}\cdot\text{d})$ at pH 7.0 and 10.4, 10.1 and $10.5 \text{ mg}/(\text{g VSS}\cdot\text{d})$ at pH 6.0 with an EDTA:Fe(II) ratio of 2.0, 1.0 and 0.5, respectively. The specific nitrate removal rate increased from 4.7 to $6.1 \text{ mg}/(\text{g VSS}\cdot\text{d})$ by decreasing the EDTA:Fe(II) ratio from 2.0 to 1.0 with the pure *T. denitrificans* culture at pH 7.0. A similar trend was observed at pH 6.0, with

the specific nitrate removal rate increasing from 2.4 to 2.6 and 3.3 mg/(g VSS·d) with an EDTA:Fe(II) ratio of 2.0, 1.0 and 0.5, respectively.

The specific nitrate removal rate was 4.3 and 4.9 mg/(g VSS·d) for activated sludge inoculum at pH 7.0 and 6.0, respectively, with an EDTA:Fe(II) ratio of 2.0. An increase of the nitrate removal rate by 9 and 29% was observed by decreasing the EDTA:Fe(II) ratio to 1.0 at pH 7.0 and 6.0, respectively. The highest nitrate removal rate was obtained with an EDTA:Fe(II) ratio of 0.5, i.e. 4.8 and 5.2 mg/(g VSS·d) at pH 7.0 and 6.0, respectively.

The most significant effect of the EDTA concentration was observed with the *Pseudogulbenkiania* strain 2002 culture, indicating the lower tolerance of this species to EDTA. The lowest molar EDTA:Fe (II) ratio resulted in an almost double nitrate removal rate compared to that achieved with an EDTA:Fe(II) ratio of 2.0. The specific nitrate removal rate increased from 1.8 to 2.5 and 3.3 mg/(g VSS·d), with a decreasing EDTA:Fe(II) ratio. A less significant increase was observed at pH 6.0, with nitrate removal rates of 1.7, 2.2 and 2.5 mg/(g VSS·d) with an EDTA:Fe(II) ratio of 2.0, 1.0 and 0.5, respectively.

The decrease of feed EDTA from 20 to 5 mM resulted in an increase of Fe(II) oxidation by 48, 14, and 49% at pH 7.0 in the experiments with *Thiobacillus*-mixed, activated sludge and *Pseudogulbenkiania* strain 2002 cultures, respectively. Fe(II) oxidation increased by 12, 17 and 23% in the experiments with *Thiobacillus*-mixed, pure *T. denitrificans* and activated sludge cultures, respectively, at pH 6.0. A decrease of 10% was observed at pH 7.0 for the pure *T. denitrificans* culture, whereas Fe(II) oxidation was 48% lower at pH 6.0 for *Pseudogulbenkiania* strain 2002. The Fe(II) oxidation rate was not significantly affected by the decreasing EDTA concentrations at pH 6.0 in the other microbial cultures. Denitrification was also maintained at the lowest EDTA:Fe(II) ratio, indicating that the low EDTA efficiently chelated Fe(II) and promoted its bioavailability.

This study demonstrated that all the investigated cultures were alleviated by the decrease of EDTA. Free EDTA is generally the most toxic EDTA form to bacteria as it disrupts the cell membranes (Oviedo and Rodríguez, 2003). However, Chakraborty and Picardal (2013) reported a negligible amount of free EDTA at an EDTA:Fe(II) ratio of 2.0, simulated by the Visual MINTEQ software. At a lower extent, EDTA toxicity can also be associated with chelated Fe(II)-EDTA and Fe(III)-EDTA species (Klueglein *et al.*, 2015). In this study, the mechanism of inhibition by EDTA at the higher EDTA:Fe(II) ratios remains

unclear. In spite of this, a faster metabolic activity was observed during the enrichment of all cultures.

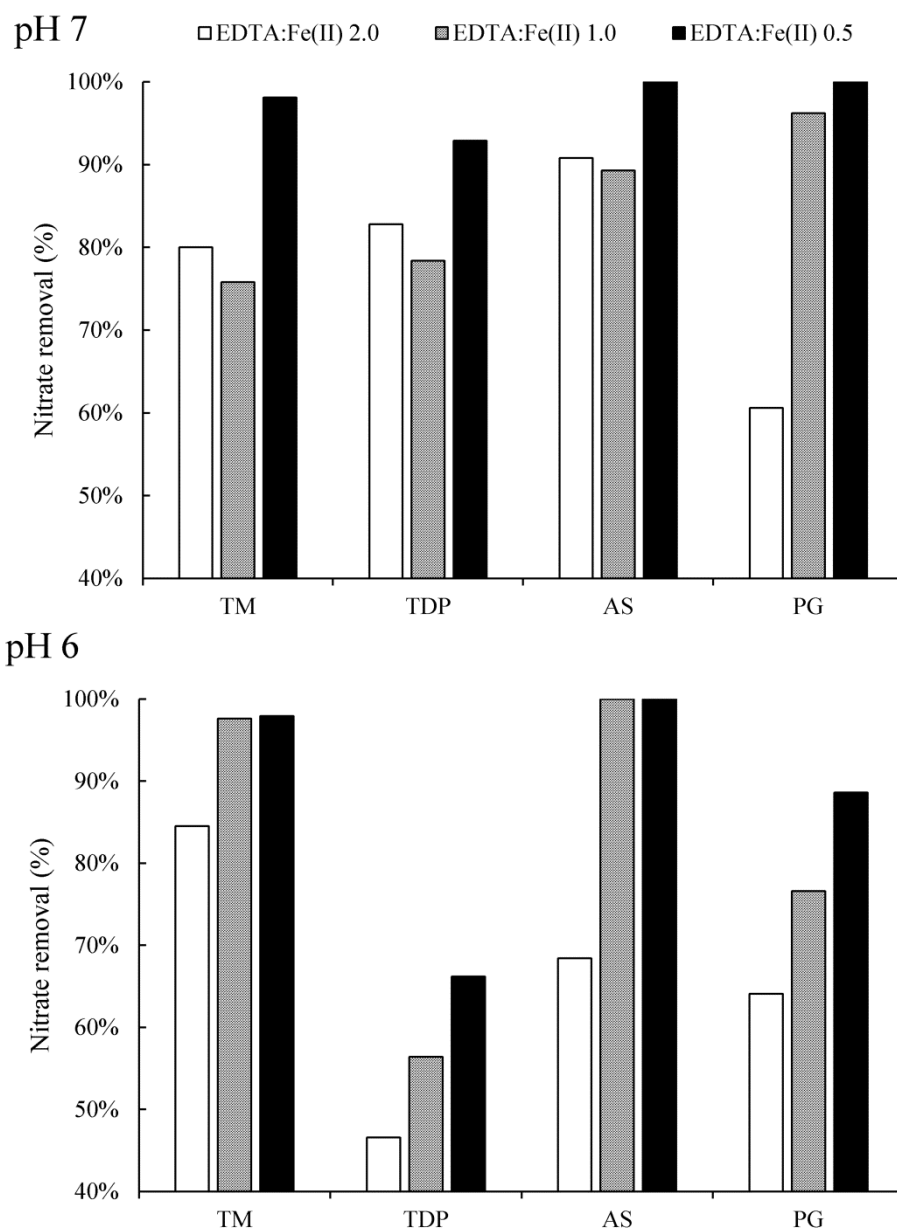


Figure 3.3: Nitrate removal obtained with TM, TDP, AS and PG in Experiment 4 at pH 7.0 and 6.0 with EDTA:Fe(II) ratios of 2.0, 1.0 and 0.5.

3.3.3 Influence of the initial pH

The effect of decreasing pH from 7.0 to 6.0 on Fe(II)-mediated autotrophic denitrification was investigated in Experiment 4. A further pH decrease to 5.0 was assessed

with the *Thiobacillus*-mixed, pure *T. denitrificans* and activated sludge cultures in Experiment 5.

It is known that pH affects microbial activity and iron speciation (Hedrich *et al.*, 2011). At pH < 4.0, Fe(II) is more stable but the inhibition of most denitrifiers occurs. Conversely, a neutral pH is favorable for biological activity but Fe(II) is quickly oxidized with oxygen (Johnson *et al.*, 2012). The known Fe(II)-oxidizing denitrifiers are neutrophilic (Hedrich *et al.*, 2011), and therefore up to now the majority of studies was performed at pH between 6.0 and 8.0 (Kiskira *et al.*, 2017).

Studies investigating the optimal pH on the process with pure and mixed cultures reported that pH should not be below 6.0 (Straub *et al.*, 2004; Oshiki *et al.*, 2013; Zhang *et al.*, 2015). Only some uncultured *Actinobacteria* were found to be capable of performing Fe(II)-driven denitrification at pH 4.5 (Kanaparthi *et al.*, 2013).

In this study, the activity of the *Thiobacillus*-mixed and activated sludge enrichments was enhanced by decreasing pH from 7.0 to 6.0 (Fig. 3.3). With an EDTA:Fe(II) ratio of 2.0, the specific nitrate removal rate increased from 8.8 to 10.4 mg/(g VSS·d) for the *Thiobacillus*-mixed culture. A slight increase of denitrification efficiency was also observed for the activated sludge inoculum, with the nitrate removal rate increasing from 4.8 mg/(g VSS·d) at pH 7.0 to 5.2 mg/(g VSS·d) at pH 6.0, with an EDTA:Fe(II) ratio of 0.5. Nielsen and Nielsen (1998) reported a more significant pH dependence of an activated sludge inoculum, with an optimal pH of 8.0. At pH 8.0, the Fe(II) oxidation rate was 0.132 mM Fe(II)·(g VSS·h)⁻¹, which was 2 times higher than that at pH 7.0, and almost 4 times higher than that at pH 6.0 and 5.0. Nielsen and Nielsen (1998) did not perform any previous enrichment on Fe(II), most likely inducing a higher pH dependence of Fe(II)-mediated denitrification.

In this study, the two pure cultures of *T. denitrificans* and *Pseudogulbenkiania* strain 2002 demonstrated to be less tolerant to decreasing pH. The decrease of pH from 7.0 to 6.0 resulted in a lower denitrification efficiency in the experiments with *Pseudogulbenkiania* strain 2002, with all the EDTA:Fe(II) ratios tested. In agreement, Weber *et al.* (2009) reported a faster growth of *Pseudogulbenkiania* strain 2002 at a pH ranging between 6.8 and 8.0. A significant decrease of nitrate removal rate was also observed for the *T. denitrificans* culture at pH 6.0. The nitrate removal rate dropped from 4.7, 6.1 and 5.9 to 2.4, 2.6 and 3.3 mg/(g VSS·d) by decreasing pH from 7.0 to 6.0 with an EDTA:Fe(II) ratio of 2.0, 1.0 and

0.5, respectively. The optimal pH for maintaining sulfur-driven denitrification with *T. denitrificans* is approximately 6.9 (Kelly and Wood, 2000). A better activity of *T. denitrificans* at neutral pH was confirmed in this study, by using Fe(II) as electron donor. However, to the best of the authors' knowledge, no information concerning the optimal pH in Fe(II)-mediated denitrification by *T. denitrificans* had previously been reported.

The effect of pH 5.0 with an EDTA:Fe(II) molar ratio of 0.5 was also investigated for the *Thiobacillus*-mixed, pure *T. denitrificans* and activated sludge cultures in Experiment 5 (Fig. 3.4). *Pseudogulbenkiania* strain 2002 was not tested at pH 5.0, as nitrate removal rate was significantly low at pH 6.0. The efficiency of denitrification considerably decreased at pH 5.0 for all the microbial enrichments. The specific nitrate removal rate dropped from 10.5, 3.3 and 5.2 mg/(g VSS·d) to 3.7, 1.5 and 1.5 mg/(g VSS·d), by decreasing the pH from 6.0 to 5.0 with the *Thiobacillus*-mixed, pure *T. denitrificans* and activated sludge cultures, respectively.

Both chemical and biological Fe(II) oxidation coupled to denitrification were repressed at decreasing pH, resulting in lower Fe(II) oxidation rates in almost all the experiments.

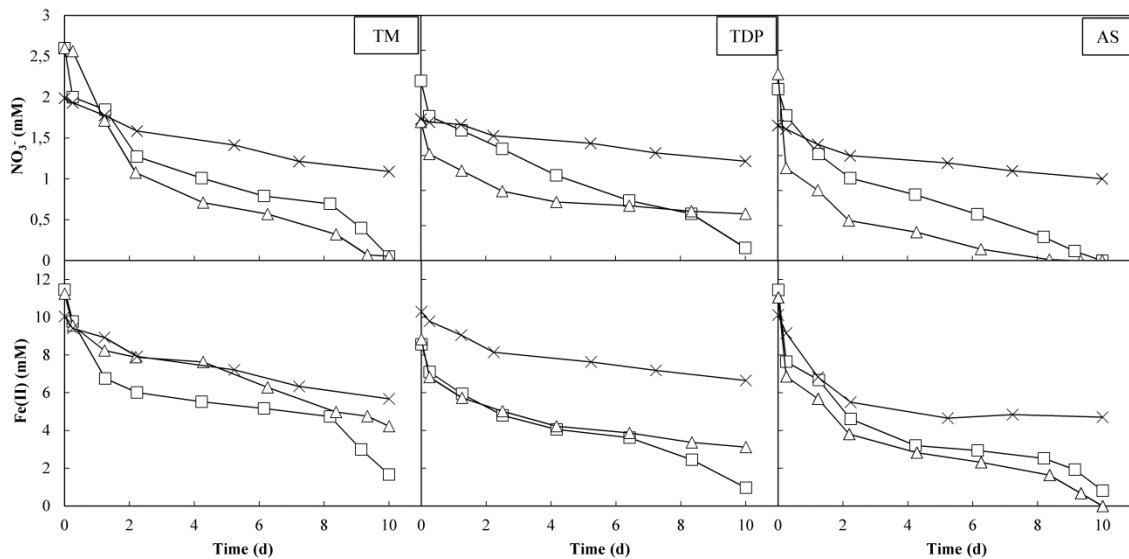


Figure 3.4: Fe(II) and NO₃⁻ profiles for TM, TDP and AS at pH 7.0 (□), 6.0 (Δ) and 5.0 (x) in Experiment 5. Standard deviations are in the range 0.02-0.60 mM.

3.4 Conclusions

Fe(II)-mediated autotrophic denitrification was effectively maintained with two pure and two enriched mixed cultures. Nitrate removal was above 60% in all the bioassays operated at pH 7.0 and an EDTA:Fe(II) ratio of 2.0. After a longer acclimation to Fe(II) and stimulation with $\text{S}_2\text{O}_3^{2-}$, the *Thiobacillus*-mixed culture resulted in the highest specific nitrate removal rate, equal to 8.8 mg/(g VSS·d). Decreasing EDTA:Fe(II) ratios resulted in higher nitrate removal efficiency and rates. With EDTA:Fe(II) ratios of 1.0 and 0.5, denitrification was particularly enhanced for *Pseudogulbenkiania* strain 2002, which less tolerated EDTA. At pH 6.0, the activity of *T. denitrificans* and *Pseudogulbenkiania* strain 2002 was repressed, whereas a faster denitrification was observed for the *Thiobacillus*-mixed and AS cultures. The use of pH 5.0 resulted in a 65, 75 and 69% slower nitrate removal than at pH 7.0 for the *Thiobacillus*-mixed, pure *T. denitrificans* and activated sludge cultures, respectively.

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CHAPTER 4

Effect of copper, nickel and zinc on Fe(II)- mediated autotrophic denitrification

4.1 Introduction

Nitrate is a common pollutant in municipal and industrial wastewaters. The extensive use of nitrogen-based fertilizers and chemicals is among the main reasons of nitrate contamination of water bodies (Viers et al., 2012). Moreover, nitrate often co-occurs with metals and heavy metals (HMs), such as iron, copper, cobalt and nickel in mining water streams (Papirio et al., 2014; Zou et al., 2014). The presence of HMs in the water resources is a major concern (Ochoa-Herrera et al., 2011; Santos and Judd, 2010), with several industrial activities being mostly responsible for their discharge in the hydrosphere (Karvelas et al., 2003).

The microbial activity of microorganisms capable for denitrification can be either stimulated or inhibited by the presence of HMs. The supplementation of trace metals can enhance metabolic degradation, whereas the excess of metals usually represses the activity of most denitrifiers (Gikas, 2007). Microbes can tolerate increasing metal concentrations by developing particular mechanisms such as the efflux of metal ions outside the cell, the accumulation and complexation of the metal ions inside the cell, and the reduction of the HM ions to a less toxic state (Spain, 2003). However, at higher concentrations, metals can change the microbial enzyme conformation and block essential functional groups (Giller et al., 2009). Besides the amount and type of HM, the toxicity of HMs can be influenced by many factors such as the temperature, the presence of specific chemical substances, the metal speciation and bioavailability, and the concomitant presence of other HMs or major elements such as calcium (Gikas, 2008).

Copper (Cu) and zinc (Zn) are among the most common polluting metal ions in industrial effluents and are associated with toxicity issues. In experiments with anoxic-membrane bioreactors, the presence of Cu and Zn at a concentration of approximately 2.6 and 32.3 mg/L repressed the denitrification rate by 20 and 34%, respectively (Feng et al., 2013). In contrast, nickel (Ni) has been found to enhance the growth of microorganisms at concentrations below 5 mg Ni/L (Gikas, 2008), and denitrifiers have been observed to tolerate Ni concentrations up to 500 mg Ni/L (Zou et al., 2015). A longer exposure of the bacteria to metals can increase their metal tolerance, thus promoting denitrification (Sakadevan et al., 1999; Zou et al., 2015).

The effect of HMs has been mostly investigated on nitrifying (Hu et al., 2004; Lee et al., 2009) and heterotrophic denitrifying microbial cultures (Lawrence et al., 2004; Ochoa-

Herrera et al., 2011; Sakadevan et al., 1999; Wang et al., 2013). However, the knowledge about the metal influence on Fe(II)-oxidizing autotrophic denitrifiers remains limited (Kiskira et al., 2017a). Hohmann et al. (2009) studied the effect of arsenic(III) as inhibiting element and reported that the enriched cultures of *Acidovorax* strain BoFeN1, *Rhodobacter ferrooxidans* strain SW2 and the enrichment culture KS tolerated up to 20-50 mM of As(III), resulting in a complete Fe(II) oxidation. To the authors' knowledge, the impact of Cu, Ni and Zn as inhibitors of Fe(II)-mediated autotrophic denitrification and the possibility of precipitating them with the biogenic Fe(III) hydro(oxides) have not yet been investigated.

Therefore, the objectives of this work were 1) to investigate the efficiency and specific rates of nitrate removal during Fe(II)-mediated autotrophic denitrification in the presence of Cu, Ni and Zn in batch bioassays, and 2) to evaluate how four different microbial cultures, i.e. a *Thiobacillus*-dominated mixed culture, an activated sludge enrichment, and two pure cultures of *Pseudogulbenkiania* sp. 2002 and *T. denitrificans*, tolerated the presence of Cu, Ni and Zn.

4.2 Materials and methods

4.2.1 Sources of microorganisms and mineral growth media

Four chemolithotrophic denitrifying cultures were used in this study. A *Thiobacillus*-dominated mixed culture, developed in experiments with thiosulfate as electron donor (Di Capua et al., 2016; Zou et al., 2016), and an activated sludge enrichment, originally collected from the municipal wastewater plant in Cassino (Italy), were used as mixed bacterial consortia. Then, two pure cultures of *Pseudogulbenkiania* sp. 2002 (DSM 18807) and *T. denitrificans* (DSM 12475) were obtained from the 'Leibniz-Institute DSMZ - German collection of microorganisms and cell cultures' in Braunschweig (Germany). Prior to this study, all the cultures had been used in Fe(II)-based denitrification bioassays (Kiskira et al., 2017b).

The basal medium was prepared with the following components (g/L): 2.00 NaHCO₃, 0.25 NH₄Cl, 0.30 KH₂PO₄, 0.40 K₂H₂PO₄, and 0.10 NaCl. The trace mineral solution was added from a sterile stock solution as detailed in Weber et al. (2009). The feed Fe(II) and NO₃⁻ concentrations were 600 and 120 mg/L, respectively.

4.2.2 Preparation of the experiments

Serum bottles of 125 mL were used for the batch experiments and maintained at room temperature ($22 \pm 2^\circ\text{C}$). Fe(II) and NO_3^- were added in concentration of 600 and 120 mg/L, in the form of iron(II) chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) and sodium nitrate (NaNO_3), respectively. EDTA in a molar ratio of 0.5:1.0 with Fe(II) was used as chelating agent. Cu, Ni and Zn were added in concentration of 5, 10, 20 and 40 mg/L in the form of copper chloride (CuCl_2), nickel chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) and zinc chloride (ZnCl_2). All the chemicals were of analytical grade (Sigma Aldrich, Germany).

The feed pH was adjusted to 7.0 by adding NaOH and HCl before flushing the bottles with He in order to maintain anoxic conditions. Bicarbonate (2 g/L as NaHCO_3) was added to each bottle as pH buffer and inorganic carbon source. Both mixed and pure cultures were taken from the liquor of the bioassays of a previous experiment (experiment 4 in Kiskira et al., 2017b), which was properly stored at 4°C for 1 month prior to performing the experiments of the present study. All the cultures were seeded in the serum bottles in a 10% (v/v) amount. The initial volatile suspended solids (VSS) concentration was 200, 320, 410 and 750 mg VSS/L in the bottles inoculated with the *Thiobacillus*-mixed culture, *T. denitrificans* pure culture, activated sludge enrichment and *Pseudogulbenkiania* sp. 2002, respectively. Then, the bottles were sealed with butyl rubber stoppers and aluminum crimps and placed on a gyratory shaker at 220 rpm. Microcosms were prepared in duplicate. For each microbial culture, controls without electron donors and metals were carried out to monitor the removal of NO_3^- , which was not associated with chemolithotrophic denitrification. Abiotic controls were also performed for possible chemical reactions between Fe(II), NO_3^- and/or Cu, Ni and Zn.

4.2.3 Thermodynamic modeling of metal speciation in the batch experiments

Metal speciation was predicted using Visual MINTEQ ver.3.1 (KTH, SEED, Sweden), a thermodynamic equilibrium modeling software (<http://vminteq.lwr.kth.se/>). Visual MINTEQ allows for simulating chemical processes, while it is not capable for taking into account biological processes (e.g. biodegradation and biosorption). However, the modeling by Visual MINTEQ can give important information on the metal speciation based on the experimental observations associated with a biological process. In this study, the

dissolved and precipitated metal concentrations were simulated for the experiments performed with the *Thiobacillus* mixed culture as well as the abiotic control by using the concentrations measured after 4 days of incubation as input data. The simulations were performed accounting for Fe(II) and Fe(III), EDTA, nutrients, trace element concentrations as reported in section 2.1, and 40 mg/L of added Cu, Ni and Zn. The temperature was set to 22°C, the pH was fixed at 6.3 and the oversaturated solids were allowed to precipitate.

4.2.4 Calculations

Nitrate removal and Fe(II) oxidation efficiencies were calculated based on the following equations:

$$\text{Nitrate removal efficiency [\%]} = \frac{\text{Initial } NO_3^- [\text{mg/L}] - \text{Final } NO_3^- [\text{mg/L}]}{\text{Initial } NO_3^- [\text{mg/L}]} \times 100$$

$$\text{Fe(II) oxidation efficiency [\%]} = \frac{\text{Initial Fe(II)} [\text{mg/L}] - \text{Final Fe(II)} [\text{mg/L}]}{\text{Initial Fe(II)} [\text{mg/L}]} \times 100$$

Nitrate removal and Fe(II) oxidation specific rates were calculated along the 10 days of each experiment as follows:

$$\text{Specific nitrate removal rate} = \frac{\text{Initial } NO_3^- [\text{mg/L}] - \text{Final } NO_3^- [\text{mg/L}]}{\text{Initial VSS} \left[\frac{\text{gVSS}}{\text{L}} \right] \cdot \text{duration [d]}}$$

$$\text{Specific Fe(II) oxidation rate} = \frac{\text{Initial Fe(II)} [\text{mg/L}] - \text{Final Fe(II)} [\text{mg/L}]}{\text{Initial VSS} \left[\frac{\text{gVSS}}{\text{L}} \right] \cdot \text{duration [d]}}$$

Inhibition of denitrification by Cu, Ni and Zn was calculated as reported below:

$$\text{Inhibition [\%]} = NO_3^- \text{ removal efficiency without metals} - NO_3^- \text{ removal efficiency with each metal}$$

4.2.5 Statistical analysis

Statistically significant differences between the nitrate removal with and without the addition of Cu, Ni and Zn in batch bioassays were determined by one-way ANOVA analysis, using the Excel statistical package. The results were considered statistically significant when the p-value was below 0.05.

4.2.6 Sampling and analytical methods

Ferrous iron, nitrate, nitrite (NO_2^-), HMs (Cu, Ni, Zn) and pH were analyzed at $t=0$, after 6 h on day 1 and, subsequently, every 24 h till day 10. Samples were taken with 5-mL disposable syringes. The NO_3^- and NO_2^- concentrations were analyzed by ion chromatography (IC) with chemically suppressed conductivity using an ICS-1100 system (ThermoFisher Scientific, USA) equipped with an AERS 500 Carbonate Electrolytically Regenerated Suppressor, 2 mm, an IonPac AG15 - 9 μm pre-column, an IonPac AS15 -9 μm column and an AS-DV autosampler. The liquid samples were filtered with 0.22 μm syringe cellulose membranes (EMD Millipore, USA) prior to IC analysis. Ferrous iron was quantified photometrically by using an UV-1800 240V IVDD UV spectrophotometer (Shimadzu, Japan), following the analytical method with 1,10-phenanthroline reported by Ahoranta et al. (2016). Fe(II) determination was performed immediately after the sampling, for avoiding chemical Fe(II) oxidation. The filtered samples were acidified using 0.5% HNO_3 and stored for analyses of the Cu, Ni and Zn concentrations by inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8300 Perkin Elmer, USA). The calibrations were performed using a multi-element solution (Perkin Elmer, USA) and the selected wavelengths were the following: 327, 221 and 206 nm for Cu, Ni, and Zn respectively. VSS were analyzed according to the standard methods (APHA, 1992).

4.3 Results and discussion

4.3.1 Fe(II)-mediated autotrophic denitrification efficiency in the absence of metals

Denitrification coupled to Fe(II) oxidation was initially investigated with Fe(II) as the sole electron donor in the absence of Cu, Ni and Zn at the optimal Fe(II):EDTA ratio 0.5:1, according to Kiskira et al. (2017b). In all experiments, no nitrite was detected as intermediate of denitrification and pH slightly decreased from 7.0 to 6.3. According to Visual MINTEQ, the concentrations of non-complexed EDTA were negligible (data not shown), indicating that EDTA most probably did not have an inhibitory effect towards microorganisms (Chakraborty and Picardal, 2013).

The use of the *Thiobacillus*-mixed culture resulted in a complete nitrate removal in 7 d (Fig. 1), faster than that observed by Kiskira et al. (2017b) in 10 d, and the highest specific nitrate removal rate, i.e. 10.4 mg $\text{NO}_3^-/(\text{g VSS}\cdot\text{d})$. However, the specific nitrate removal rate

was very similar to that achieved in the previous study due to a 11% higher VSS concentration (Table 4.1).

Fe(II)-based denitrification was also studied with a *T. denitrificans* pure culture. The specific nitrate removal rate was 4.4 mg NO₃⁻/(g VSS·d), i.e. approximately 55% lower compared to that obtained with the *Thiobacillus*-mixed culture (Table 1). Nitrate removal efficiency reached 91% in 10 d with the *T. denitrificans* pure culture (Fig. 1), thus similar to that reported by Kiskira et al. (2017b). The specific Fe(II) oxidation rate, i.e. 17.8 mg Fe(II)/(g VSS·d), was slightly higher than the 17.0 mg Fe(II)/(g VSS·d) observed in the previous study. A similar trend was also observed with the activated sludge enrichment. The specific nitrate removal rate was 3.7 mg NO₃⁻/(g VSS·d) and the specific Fe(II) oxidation rate increased from 12.0 to 17.5 mg Fe(II)/(g VSS·d), in comparison with Kiskira et al. (2017b). Nitrate removal and Fe(II) oxidation efficiencies reached up to 96 and 92% respectively, after 10 d (Fig. 4.1).

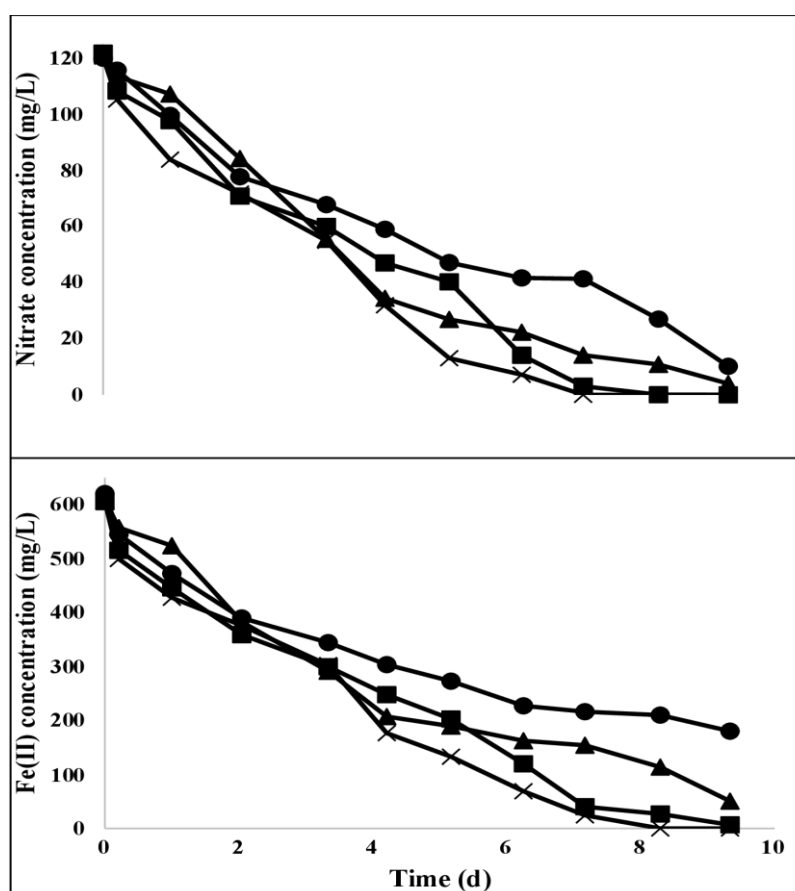


Figure 4.1: Nitrate and Fe(II) evolution during autotrophic denitrification with the *Thiobacillus* mixed culture (■), activated sludge enrichment (▲), *T. denitrificans* (●) and *Pseudogulbenkiania* sp. 2002 (x) pure cultures. No Cu, Ni and Zn was added. Standard deviations are in the range 0.2-18.4 mg/L

Table 4.1: Inhibition (%) of denitrification, specific nitrate removal rates and specific Fe(II) oxidation rates for the *Thiobacillus* mixed culture (TM), activated sludge enrichment (AS), *T. denitrificans* (TDP) and *Pseudoglubenkiania* sp. 2002 (PG) pure cultures in the absence and presence of 5, 10, 20 and 40 mg/L of added Cu, Ni and Zn.

Initial metal concentration (mg/L)	TM		AS		TDP		PG	
	Inhibition (%)	Specific nitrate removal rate [mg NO ₃ /(g VSS·d)]	Inhibition (%)	Specific nitrate removal rate [mg NO ₃ /(g VSS·d)]	Inhibition (%)	Specific nitrate removal rate [mg NO ₃ /(g VSS·d)]	Inhibition (%)	Specific nitrate removal rate [mg NO ₃ /(g VSS·d)]
0	0	10.4	0	3.7	0	4.4	0	2.8
Copper (Cu)								
5	15	6.8	4	3.5	9	4.0	17	1.7
10	30	5.4	23	2.9	26	3.1	36	1.3
20	51	3.8	33	2.4	54	2.0	73	0.6
40	66	2.6	46	1.9	71	1.4	94	0.1
Nickel (Ni)								
5	0	7.8	0	3.6	0	4.4	25	1.5
10	5	7.4	3	3.5	13	3.9	31	1.4
20	10	7.2	5	3.5	30	3.1	38	1.3
40	34	5.2	30	2.6	43	2.5	92	0.2
Zinc (Zn)								
5	6	7.4	4	3.5	9	4.0	17	1.7
10	10	7.0	9	3.3	20	3.6	29	1.5
20	17	6.6	17	3.1	26	3.2	50	1.0
40	40	4.6	41	2.1	46	2.3	94	0.1

Table 4.2: Thermodynamic modeling by Visual MINTEQ of dissolved and precipitated metals after 4 d in the experiments with the *Thiobacillus*-mixed culture and in abiotic controls with and without the addition of 40 mg/L of Cu, Ni and Zn at pH 6.3. Values are given as concentrations (mg/L) and percentage (%) of the input data.

Input dissolved Fe(II) and Fe(III) concentration (mg/L)	Dissolved metals (mg/L)	Precipitated metals (mg/L)	Main precipitates formed
Fe(II): 259 Fe(III): 341	Fe(II): 159 (61%) Fe(III): 145 (43%)	Fe (II): 100 (39%) Fe(III): 195 (57%)	Fe(II) species: Siderite and Vivianite Fe(II) and Fe(III) species: Magnetite, $\text{Fe}_3(\text{OH})_8$ Fe(III) species: CoFe_2O_4 , Cupric ferrite, $\text{Fe}(\text{OH})_2 \cdot 7\text{Cl}_3$, Ferrihydrite, Goethite, Hematite, (H/K/Na)-Jarosite, Lepidocrocite, Maghemite, Magnesioferrite, Strengite
Fe(II): 470 Fe(III): 130 Cu: 40	Fe(II): 300 (64%) Fe(III): 78 (60%) Cu: 20 (50%)	Fe (II): 169 (36%) Fe(III): 52 (40%) Cu: 20 (50%)	Fe(II) species: Siderite and Vivianite Fe(II) and Fe(III) species: Magnetite, $\text{Fe}_3(\text{OH})_8$ Fe(III) species containing Cu: Cupric ferrite Fe(III) species: CoFe_2O_4 , $\text{Fe}(\text{OH})_2 \cdot 7\text{Cl}_3$, Ferrihydrite, Goethite, Hematite, Hercynite, (K/Na)-Jarosite, Lepidocrocite, Maghemite, Magnesioferrite, Strengite Cu species: Atacamite, Azurite, Brochantite, $\text{Cu}_3(\text{PO}_4)_2$, $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$, CuCO_3 , Malachite, Tenorite
Fe(II): 578 Fe(III): 22 Cu: 40 No biomass	Fe(II): 403 (70%) Fe(III): 21 (97%) Cu: 36 (90%)	Fe (II): 173 (30%) Fe(III): 1 (3%) Cu: 4 (10%)	Fe(II) species: Siderite and Vivianite Fe(II) and Fe(III) species: Magnetite Fe(III) species containing Cu: Cupric ferrite Fe (III) species: CoFe_2O_4 , $\text{Fe}(\text{OH})_2 \cdot 7\text{Cl}_3$, Ferrihydrite, Goethite, Hematite, Hercynite, Lepidocrocite, Maghemite, Magnesioferrite, Strengite
Fe(II): 358 Fe(III): 242 Ni: 40	Fe(II): 218 (61%) Fe(III): 141 (58%) Ni: 20 (90%)	Fe (II): 100 (39%) Fe(III): 101 (42%) Ni: 20 (50%)	Fe(II) species: Siderite and Vivianite Fe(II) and Fe(III) species: $\text{Fe}_3(\text{OH})_8$, Magnetite Fe (III) species: CoFe_2O_4 , Cupric ferrite, $\text{Fe}(\text{OH})_2 \cdot 7\text{Cl}_3$, Ferrihydrite, Goethite, Hematite, (K/Na)-Jarosite, Lepidocrocite, Maghemite, Magnesioferrite, Strengite Ni species: $\text{Ni}_3(\text{PO}_4)_2$ and NiCO_3
Fe(II): 343 Fe(III): 257 Zn: 40	Fe(II): 212 (63%) Fe(III): 143 (57%) Zn: 20 (50%)	Fe (II): 130 mg/L (37%) Fe(III): 113 mg/L (43%) Zn: 20 mg/L (50%)	Fe(II) species: Siderite and Vivianite Fe(II) and Fe(III) species: $\text{Fe}_3(\text{OH})_8$, Magnetite Fe(III) species: CoFe_2O_4 , Cupric ferrite, $\text{Fe}(\text{OH})_2 \cdot 7\text{Cl}_3$, Ferrihydrite, Goethite, Hematite, (K/Na/H)-Jarosite, Lepidocrocite, Maghemite, Magnesioferrite, Strengite Zn species: Smithsonite, $\text{Zn}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$, ZnCO_3 , $\text{ZnCO}_3 \cdot \text{H}_2\text{O}$

The lowest specific nitrate removal rate of 2.8 mg NO_3^- /(g VSS·d) was observed in the bioassays with the *Pseudogulbenkiania* sp. 2002. However, the specific Fe(II) oxidation rate significantly increased from 5.6 to 13.9 mg Fe(II)/(g VSS·d), comparing to what obtained by Kiskira et al. (2017b). Complete nitrate removal and Fe(II) oxidation were observed in 7 d (Fig. 4.1), indicating that the *Pseudogulbenkiania* strain was significantly enriched in comparison to the previous study, where a complete nitrate removal occurred in 10 d and only 53% of Fe(II) oxidation was recorded.

Fe(II)-mediated autotrophic denitrification proceeded in a good agreement with the stoichiometry when the *Thiobacillus*-mixed culture and *Pseudogulbenkiania* strain were used. The molar Fe(II): NO_3^- ratio was in the range 4.5-5.6, thus very similar to the theoretical value of 5.0 (Sorensen, 1987). A slightly higher molar Fe(II): NO_3^- ratio up to 6.5 was observed during the experiments with the *T. denitrificans* pure culture and activated sludge enrichment, indicating that part of the Fe(II) oxidized was not associated with denitrification. Visual MINTEQ confirmed that part of the added Fe(II) (i.e. approximately 39%) precipitated as siderite and vivianite and was not used for denitrification (Table 4.2).

In comparison with the classical heterotrophic denitrification and the sulfur-driven autotrophic denitrification, Fe(II)-mediated autotrophic denitrification remains a less efficient technology, as it results in lower nitrate removal rates (Di Capua et al., 2015). Research efforts are required in order to optimize the process by further enriching the microbial cultures on Fe(II) or using continuous-flow conditions, which can enhance denitrification efficiencies and rates (Zhang et al., 2015).

4.3.2. Influence of heavy metals on Fe(II)-mediated autotrophic denitrification

4.3.2.1 Speciation and fate of Cu, Ni and Zn

This study also evaluated the efficiency and rates of nitrate removal during Fe(II)-mediated autotrophic denitrification in the presence of Cu, Ni and Zn in batch experiments. A decrease of the soluble Cu, Ni and Zn concentration occurred in the first 4 d in all the experiments (Fig. 4.2), likely due to metal precipitation, co-precipitation, sorption onto iron (hydr)oxides, and probably sorption onto biomass (Crane et al., 2010; Kiskira et al., 2017a). The decrease was in the range 23-59% in the bottles with Zn and Cu, whereas the soluble Ni concentration decreased in the range 37-59%.

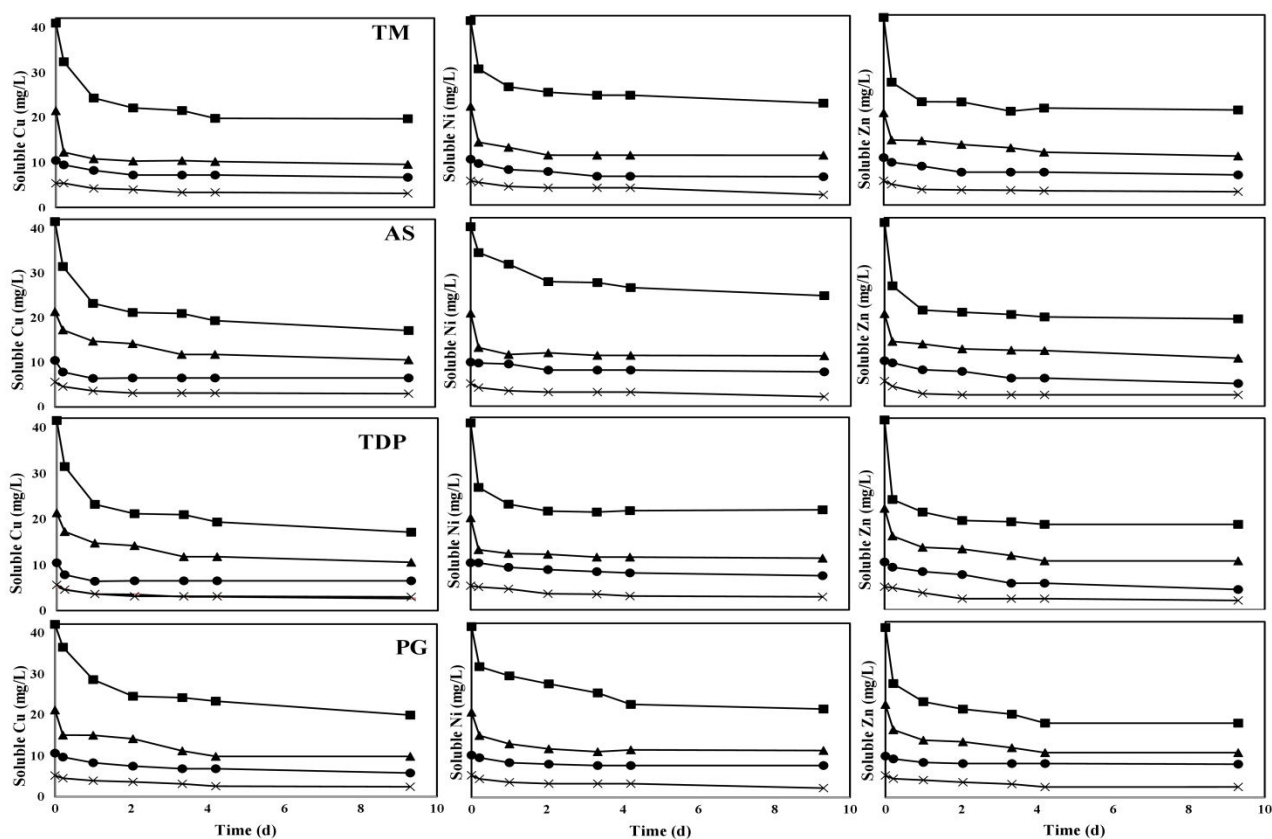


Figure 4.2: Soluble Cu, Ni, Zn profiles using the four microbial cultures with initial Cu, Ni and Zn concentrations of 5 (x), 10 (●), 20 (▲) and 40 (■) mg/L. Standard deviations are in the range 0.1-6.6 mg/L.

In agreement, the simulations performed with Visual MINTEQ indicated that approximately half of the added amount of Cu, Ni and Zn (40 mg/L) precipitated when the *Thiobacillus*-mixed culture was used (Table 4.2). The same simulations were obtained with the other microbial cultures (data not shown). The results of the simulation performed without accounting for the biomass at initial 40 mg Cu/L showed that the amount of precipitated Cu was only 4 mg/L (Table 4.2), i.e. 80% lower than that observed in the samples with the biomass. At the same time, the precipitated Fe(III) was only 3 mg/L (Table 4.2), in comparison with the 52 mg Fe(III)/L obtained with the *Thiobacillus* dominated mixed culture. This indicates that the biological Fe(II) oxidation resulted in a higher production of Fe(III) (hydr)oxides, with precipitation of Cu (as copper phosphate, copper carbonate or copper oxide) being the main mechanism for Cu removal according to Visual MINTEQ. However, also biosorption and copper co-precipitation with iron oxides most likely contributed to the decrease of soluble Cu, but Visual MINTEQ could not account for such removal processes. Similar results were obtained with Ni and Zn (Table 4.2). Further investigation is needed in order to distinguish among the different ongoing mechanisms responsible for the decrease of soluble Cu, Ni and Zn concentrations.

In this line, analytical methods such as X-ray absorption and Fourier transform infrared spectroscopy as well as flow cytometry techniques can help to evaluate the effect of heavy metal aqueous species on the microbial cell structure and the tolerance of the cells to heavy metal toxicity (Lopez-Fernandez et al., 2018). However, this was beyond the scope of the present study.

4.3.2.2 Nitrate removal and Fe(II) oxidation with Cu, Ni and Zn

Fig. 4.3 shows the nitrate removal efficiency (%) achieved after 10 d in the bioassays inoculated with the four microbial cultures at different initial metal concentrations. All metals negatively affected denitrification, whereas no stimulation was observed even at the lowest Cu, Ni and Zn concentrations (Table 4.1).

The addition of Cu, Ni and Zn at the highest initial concentration of 40 mg/L resulted in 3.0, 1.5 and 1.6 times lower nitrate removal efficiencies (p-value of 0.0001, 0.0010 and 0.0006), respectively, than those obtained without metals for the *Thiobacillus*-dominated mixed culture. Fe(II) oxidation efficiency decreased by 80% when 40 mg Cu/L was fed, and approximately 55% at the highest Ni and Zn concentrations with the *Thiobacillus*-dominated mixed culture. A statistically significant decrease was also observed with the activated sludge enrichment, as 2.0, 1.4, and 1.7 times lower specific nitrate removal efficiencies (p-value of 0.001, 0.005 and 0.003) were obtained with 40 mg/L of Cu, Ni and Zn, respectively. Fe(II) oxidation efficiency was approximately 40% lower in the experiments with the activated sludge, in comparison with the *Thiobacillus* mixed culture.

In this study, Cu was observed to be the most inhibitory metal. This is in agreement with the work of Principi et al. (2006), who reported that Cu had the greatest toxic effect on an activated sludge, in experiments performed with 0.5-1.0 mg Cu/L, 0.8-5.2 mg Ni/L and 0.8-7.1 mg Zn/L. Higher Cu, Ni and Zn concentrations were used in the current study, indicating that the activated sludge was likely more tolerant to HMs toxicity, resulting in a higher denitrification activity.

In comparison with the *Thiobacillus*-mixed culture, a significantly higher sensitivity to metal toxicity was observed for the *T. denitrificans* pure culture at 40 mg/L of Cu, Ni and Zn (p-value of 0.0348, 0.0132 and 0.0365). The specific nitrate removal rates were 1.4, 2.5 and 2.3 mg NO₃⁻/(g VSS·d) (Table 1), i.e. 69, 43 and 47% (p-value of 0.0006, 0.0021 and 0.0022) lower than those achieved in the absence of metals. The addition of 40 mg Cu/L

resulted in a 68% slower Fe(II) oxidation, whereas a 41 and 45% decrease of the specific Fe(II) oxidation rate was observed with the addition of Ni and Zn.

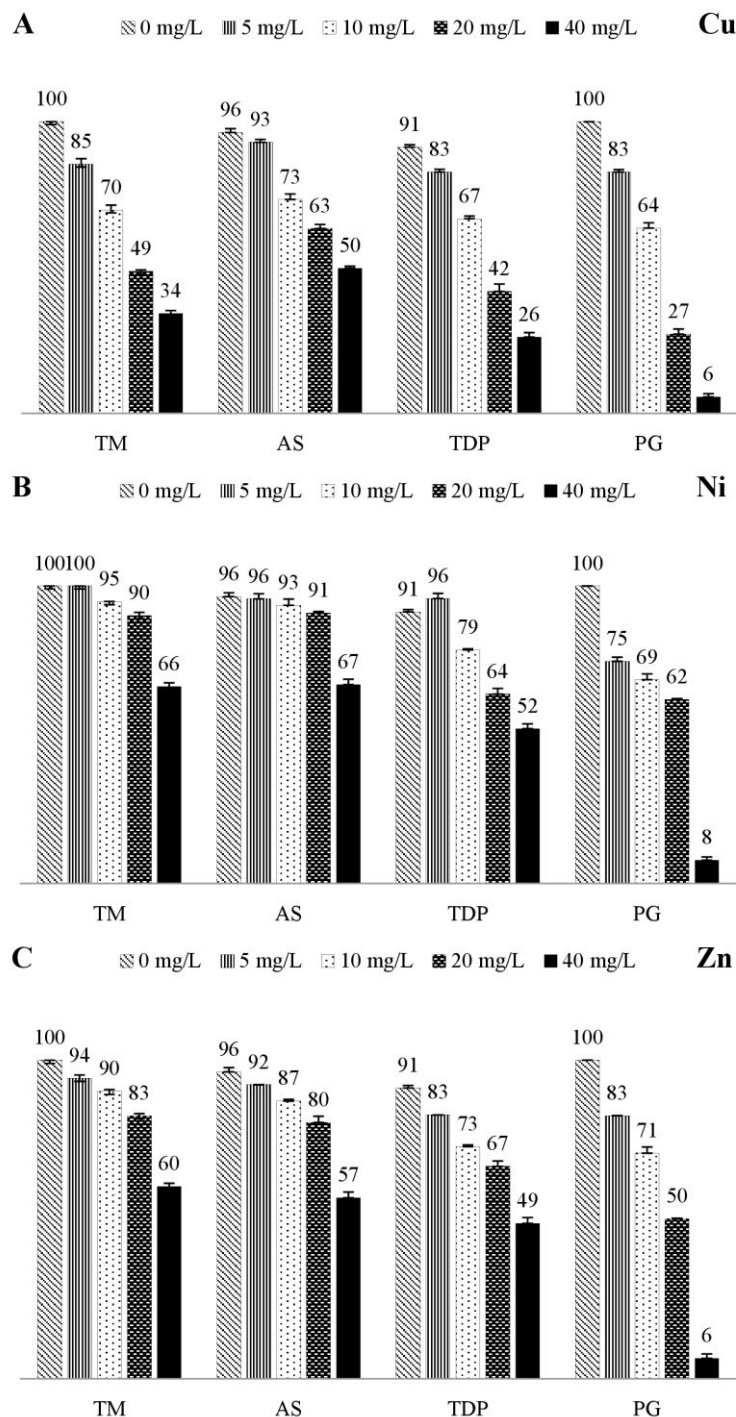


Figure 4.3: Nitrate removal efficiency (%) achieved after 10 d during Fe(II)-mediated autotrophic denitrification at different Cu (A), Ni (B) and Zn (C) initial concentrations with the *Thiobacillus*-dominated mixed culture (TM), activated sludge enrichment (AS), and two pure cultures of *T. denitrificans* (TDP) and *Pseudogulbenkiania* sp. 2002 (PG).

Pseudogulbenkiania sp. 2002 is a member of the family *Nisseriaceae* (Weber et al., 2006), which participates in the metal biogeochemical cycles. *Pseudogulbenkiania* sp. 2002 has been used for the co-precipitation of HMs with biogenic Fe(III) oxides (Weber et al., 2009). However, in this study *Pseudogulbenkiania* sp. 2002 resulted in the lowest Fe(II) mediated autotrophic denitrification efficiency when Cu, Ni and Zn were supplemented. Nitrate removal efficiency only reached 6, 8 and 6% (Fig. 4.3) when 40 mg/L of Cu, Ni and Zn, respectively, was used, in comparison with the complete NO_3^- removal achieved in the absence of metals (p-value of 0.0001, 0.0001, 0.0002). A high negative impact was observed even at an initial metal concentration of 5 mg/L, with denitrification that was inhibited by 17, 25 and 17% (Table 4.1) in the presence of Cu, Ni and Zn, respectively (p-value of 0.0022, 0.0024 and 0.0086). Up to now, only one study has been carried out on the influence of metals on the activity of *Pseudogulbenkiania* sp. 2002, with the aim of investigating As immobilization onto biogenic Fe oxyhydroxides under different initial molar Fe/As ratios (Xiu et al., 2016). Nitrate removal and Fe(II) oxidation efficiencies were not inhibited with an initial concentration of approximately 1 mg As/L, and the process efficiently resulted in the immobilization of As mainly onto biogenic lepidocrocite crystals.

4.3.2.2.1. Effect of Cu

Cu inhibited all the denitrifying cultures, in the range of initial 10-40 mg Cu/L, with the two pure cultures being more sensitive to Cu toxicity (Fig. 4.4). An initial concentration of 20-40 mg Cu/L resulted in a 73-94% and 54-71% inhibition of the *Pseudogulbenkiania* sp. 2002 and *T. denitrificans* activity, respectively. A lower inhibition was observed at the initial concentration of 5 and 10 mg Cu/L, with the denitrifying activity of *Pseudogulbenkiania* sp. 2002 and *T. denitrificans* being repressed by 17-36% (p-value of 0.0024 and 0.0032) and 9-26% (p-value of 0.0025 and 0.2023), respectively.

Denitrification was inhibited by 66% when Cu was supplemented at 40 mg Cu/L (soluble 19.7 mg Cu/L after 4 d) when the *Thiobacillus*-dominated mixed culture was used. Nitrate removal efficiency was 51, 30 and 15% lower (p-value of 0.000009, 0.0030 and 0.0160) at initial Cu concentrations of 20, 10 and 5 mg Cu/L (soluble 9.6, 6.7 and 3.1 mg Cu/L), respectively, than that obtained without supplementing Cu.

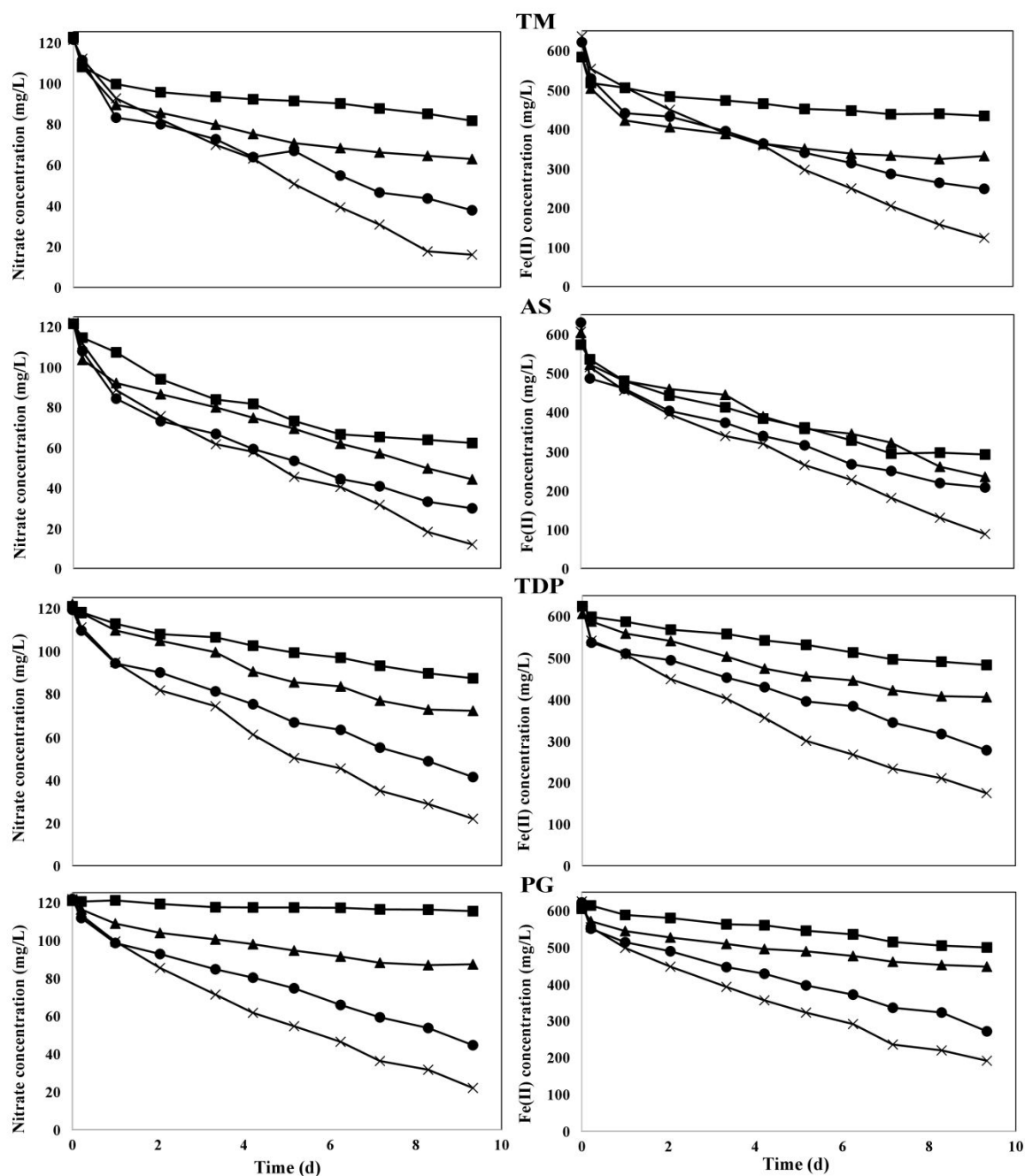


Figure 4.4: NO_3^- and Fe(II) profiles at initial 5 (x), 10 (●), 20 (▲) and 40 (■) mg Cu/L using the *Thiobacillus*-dominated mixed culture (TM), activated sludge enrichment (AS), *T. denitrificans* (TDP), and *Pseudogulbenkiania* sp. 2002 (PG) pure cultures. Standard deviations are in the range 0.1-25.0 mg/L.

The activated sludge enrichment better tolerated Cu, resulting in a 46% inhibition at the feed concentration of 40 mg Cu/L (soluble 17.1 mg Cu/L). The inhibition was 33, 23 and only 4%, when Cu was fed at the initial concentrations of 20, 10 and 5 mg Cu/L (soluble 8.7, 6.5 and 3.0 mg Cu/L), respectively. The addition of 5 mg Cu/L did not result in a statistically significant nitrate removal decrease (p-value of 0.1754), which was instead observed at 10 and 20 mg Cu/L (p-value of 0.0091 and 0.0035). Ochoa-Herrera et al. (2011) reported a

similar inhibition of nitrate removal (i.e. 50%) by Cu on denitrifiers originating from a methanogenic granular sludge at a soluble Cu concentration of approximately 1 mg Cu/L. However, the authors demonstrated that the inhibitory impact of Cu on denitrifying bacteria decreased with the exposure time. In agreement with Ochoa-Herrera et al. (2011), Zou et al. (2013) observed that the supplementation of Cu to denitrifying mixed cultures enriched on ethanol led to a 48% slower nitrate removal at a soluble Cu concentration of 0.7 mg Cu/L. In another study, Sakadevan et al. (1999) demonstrated that denitrification in soil was inhibited starting from Cu concentrations higher than 80 mg Cu/L.

4.3.2.2.2 Effect of Ni

Fig. 4.5 shows NO_3^- removal and Fe(II) oxidation at different initial Ni concentrations with all the microbial cultures investigated. Ni showed a lower inhibitory effect than Cu. Nickel has been indeed reported as an essential trace element in the chemolithotrophic growth of microorganisms, as Ni catalyzes the synthesis of hydrogenase and is a constituent of the hydrogenase in the cytoplasmic nicotinamide adenine dinucleotide (NAD) and in the membrane-bound forms (Gikas, 2008).

The activated sludge enrichment resulted only in a 0-5% inhibition at initial concentrations of 5, 10 and 20 mg Ni/L, corresponding to 2.1, 7.7 and 11.2 mg /L of soluble Ni (p-value of 0.7973, 0.3761 and 0.0610). A 30% inhibition (p-value of 0.005) of denitrification was observed at a feed concentration of 40 mg Ni/L (soluble 24.6 mg Ni/L). Different results can be found in the scientific literature, mainly due to the diverse times of exposure to Ni, operating conditions (batch or continuous-flow) or biomass agglomerates (suspended cultures or biofilms) used. For instance, Gikas (2008) reported that concentrations of about 5 mg Ni/L enhanced the activity of denitrifying microorganisms in an activated sludge, whereas Ni started to inhibit the microbial growth at concentrations higher than 10 mg Ni/L. In another study, Ni concentrations up to 27 mg Ni/L stimulated the microbial growth, while inhibition started at higher concentrations until no growth was any longer reported when the feed Ni concentration was 160 mg Ni/L (Gikas, 2007). In the current study, no stimulation was observed even at a feed concentration of 5 mg Ni/L. Similarly, Ong et al. (2004) reported inhibition of the denitrifying activity of an activated sludge at Ni concentrations up to 10 mg Ni/L.

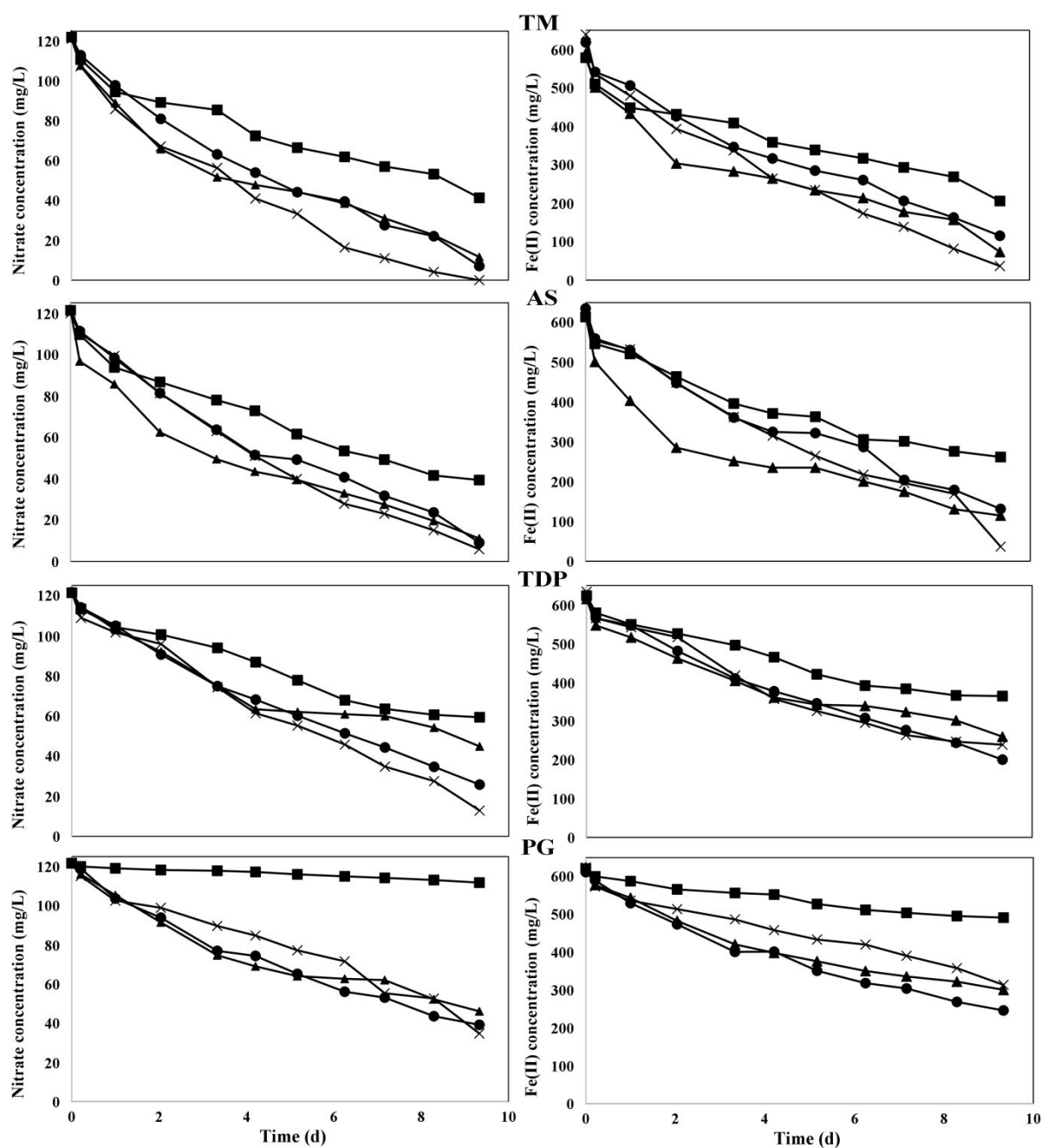


Figure 4.5: NO_3^- and Fe(II) profiles at initial 5 (x), 10 (●), 20 (▲) and 40 (■) mg Ni/L using the *Thiobacillus*-dominated mixed culture (TM), activated sludge enrichment (AS), *T. denitrificans* (TDP), and *Pseudogulbenkiania* sp. 2002 (PG) pure cultures. Standard deviations are in the range 0.2-28.7 mg/L.

Zou et al. (2014) observed that a concentration of 10 mg Ni/L did not repress denitrification of a biofilm grown onto activated carbon, originated from an activated sludge and being mainly composed by *Dechloromonas denitrificans* and *Hydrogenophaga caeni* after a long-time enrichment on ethanol. However, the nitrate removal rate decreased by 18 and 65% when Ni was fed at 50 and 100 mg Ni/L, respectively (Zou et al., 2014). In another study, Zou et al. (2015) reported that fluidized-bed denitrifying biofilms tolerated soluble Ni

concentrations up to 500 mg Ni/L, due to the enhanced tolerance of denitrifiers to Ni after approximately 250 d of incubation in continuous-flow fluidized-bed reactors (FBRs).

A low inhibition in the range 0-10 % was also observed for the *Thiobacillus*-mixed culture at initial concentrations from 5 to 20 mg Ni/L (soluble concentrations ranging from 2.3 to 11.0 mg Ni/L). The nitrate removal decrease at feed 10 and 20 mg Ni/L was statistically significant, with a p-value of 0.0003 and 0.0121, respectively. Similarly to the activated sludge enrichment, a 34% inhibition (p-value of 0.0196) of denitrification was observed at a feed concentration of 40 mg Ni/L (soluble 22.6 mg Ni/L).

An initial concentration of 5 mg Ni/L resulted in a 5% higher nitrate removal efficiency with the pure culture of *T. denitrificans* (Fig. 3), but the difference was not statistically significant (p-value 0.1419) to prove a stimulation of denitrification. At feed Ni of 10, 20 and 40 mg Ni/L, the *T. denitrificans* pure culture was significantly more affected, with the nitrate removal efficiency decreasing by 13, 30 and 43% (p-value of 0.0048, 0.0055 and 0.0020) compared to that obtained without metals. Di Capua et al. (2017) studied the inhibition by Ni on a *T. denitrificans* culture enriched on thiosulfate in FBRs. The authors reported that Ni concentrations from 25 to 100 mg Ni/L inhibited the thiosulfate-driven autotrophic denitrification by only 9-19% in batch bioassays. The higher tolerance to Ni of the denitrifying FBR cultures observed by Di Capua et al. (2017) was likely due to the use of completely developed biofilms that protected the cells from the inhibitory effects of Ni.

Pseudogulbenkiania sp. 2002 was highly affected by the presence of Ni, as only 8% of nitrate removal was observed in the batch assays when Ni was fed at 40 mg Ni/L (soluble 19.9 mg Ni/L after 4 d). Denitrification was repressed by 38, 31 and 25% at initial concentrations of 20, 10 and 5 mg Ni/L (p-value of 0.00008, 0.0037 and 0.0029), respectively.

4.3.2.2.3 Effect of Zn

The nitrate removal and Fe(II) oxidation efficiencies achieved in the presence of Zn at initial concentrations of 5, 10, 20 and 40 mg Zn/L are reported in Fig. 4.6. When Zn was supplemented at a feed concentration of 5, 10 and 20 mg Zn/L, an inhibition of nitrate removal by 6-17% (p-value of 0.0108, 0.0028 and 0.0013) and 4-17% (p-value of 0.1280, 0.0596 and 0.0213) was observed for the *Thiobacillus*-mixed culture and the activated sludge enrichment, respectively.

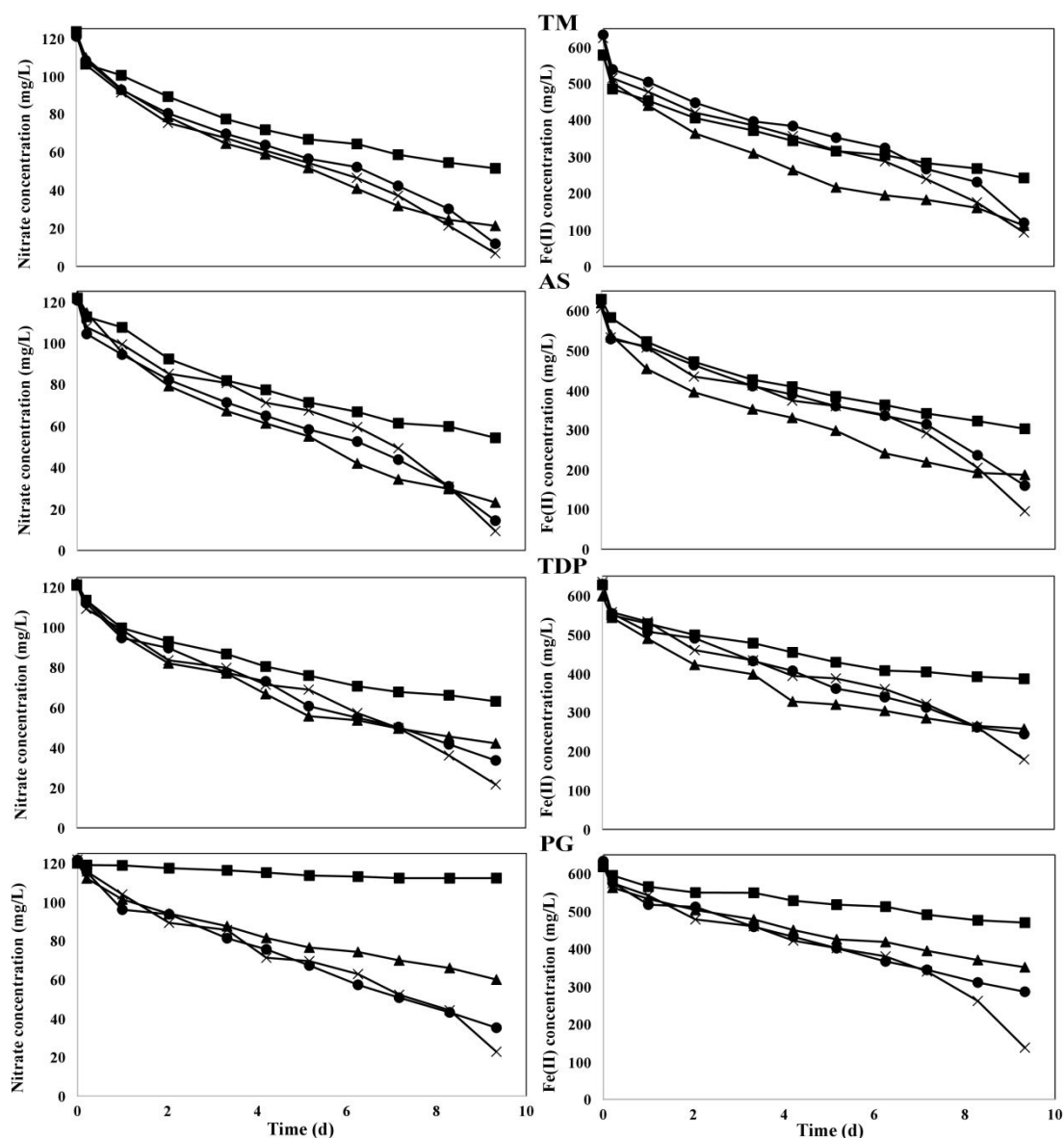


Figure 4.6: NO_3^- and Fe(II) profiles at initial 5 (x), 10 (●), 20 (▲) and 40 (■) mg Zn/L using the Thiobacillus-dominated mixed culture (TM), activated sludge enrichment (AS), *T. denitrificans* (TDP), and *Pseudogulbenkiania* sp. 2002 (PG) pure cultures. Standard deviations are in the range 0.1-21.9 mg/L.

A higher inhibition was observed for the two pure cultures. An initial concentration of 40 mg Zn/L, which corresponded to soluble 17.8 and 18.7 mg Zn/L with the *Pseudogulbenkiania* sp. 2002 and the *T. denitrificans* pure cultures, resulted in a 6 and 49% nitrate removal efficiency, respectively. At feed concentrations of 5, 10 and 20 mg Zn/L, the inhibition of nitrate removal was in the range 17-50 and 9-26% for the *Pseudogulbenkiania* sp. 2002 and the *T. denitrificans* pure culture, respectively. The nitrate removal decrease due to the presence of Zn was statistically significant at all Zn concentrations for both the pure cultures.

Sakadevan et al. (1999) reported that the addition of approximately 100 mg Zn per kg of sediment led to a stimulation of denitrification by 4%. In contrast, increasing the Zn concentration up to 300 mg/kg, a significant decrease of the denitrification rate was observed. In agreement with the present study, Feng et al. (2013) reported that the denitrification rate of a microbial community in an anoxic-membrane bioreactor decreased by 20-34% at a Zn concentration of 3.7-32.3 mg Zn/L, in the simultaneous presence of copper, lead, and cadmium.

4.4 Conclusions

The activity of the four Fe(II)-oxidizing denitrifying cultures was differently affected by the presence of Cu, Ni and Zn. Metal precipitation, co-precipitation, sorption onto iron (hydr)oxides and possible biosorption occurred, as the soluble Cu, Ni and Zn concentrations decreased up to 50% after 4 d. The *Thiobacillus*-dominated mixed culture and activated sludge enrichment better tolerated the presence of metals. In contrast, a significantly higher sensitivity to metal toxicity was observed for the pure cultures. The use of *Pseudogulbenkiania* sp. 2002 only resulted in a 6, 8 and 6% nitrate removal efficiency, when Cu, Ni and Zn were fed at 40 mg/L, respectively. Among the three HMs, Cu averagely led to the highest inhibition of denitrification.

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CHAPTER 5

Mineral characterization of the Fe(III)(hydr)oxides during Fe(II)-driven denitrification

5.1 Introduction

Fe(II)-mediated autotrophic denitrification is a promising biotechnology to be employed for iron recovery when nitrogen and metal contamination coexists (Papirio et al., 2014; Zou et al., 2015; Kiskira et al., 2017a,b). Chemoautotrophic denitrifiers are capable of coupling Fe(II) oxidation with denitrification resulting in the reduction of nitrate to nitrogen gas and the production of Fe(III) precipitates that can be subsequently removed and recovered (Kiskira et al., 2017a).

Many studies reported different biogenic Fe(III)(hydro)oxide mineral phases such as goethite, lepidocrocite, hematite, magnetite, maghemite, 2-line ferrihydrite and mixed-phase Fe(II)-Fe(III) minerals (green rust) (Senko et al., 2005a, b; Weber et al., 2006; Konhauser et al., 2011). Kiskira et al. (2017a) reviewed all biogenic Fe(III)(hydr)oxide products deriving from Fe(II)-mediated autotrophic and mixotrophic denitrification.

The use of different microorganisms results in diverse biogenic Fe(III) (oxyhydr)oxides minerals from soluble Fe(II) (Posth et al., 2014). Other factors affecting the formation of Fe(III) precipitates are the medium composition, the concentration of possible co-substrates, the incubation conditions, metabolic rates and the presence of nucleation sites (Kappler and Straub, 2005; Senko et al., 2005a; Larese-Casanova et al., 2010; Pantke et al., 2012). A slower Fe(II) oxidation produces crystalline minerals, such as goethite, while a rapid Fe(II) oxidation leads to the formation of more amorphous phases such as ferrihydrite (Lack et al., 2002; Senko et al., 2005b). pH is another factor that affects the formation and composition of Fe(III) precipitates. Goethite was reported at pH 7.7, while lepidocrocite at pH 6.3 by *Acidovorax* sp. BoFeN1 (Larese-Casanova et al., 2010).

Hennebel et al. (2009) reported that biogenic oxides are more efficient for heavy metal adsorption than chemically produced oxides, due to their larger specific surface area and higher binding energy. Arsenic has been reported to co-precipitate with the biogenic Fe(III) minerals (Senn and Hemond, 2002; Hohmann et al., 2009; Sun et al., 2009; Ahoranta et al., 2016). Microbial oxidation of As(III) and Fe(II) linked to denitrification resulted in an enhanced immobilization of As(V) onto biogenic Fe(III) (hydr)oxides in sand-packed bed columns (Sun et al., 2009). Furthermore, arsenic immobilization by biogenic goethite and lepidocrocite has also been reported (Hohmann et al., 2009; Xiu et al., 2016).

Fe(II)-mediated autotrophic denitrification has been usefully applied in recovery of co-precipitated or absorbed heavy metals (Finneran et al., 2002; Hohmann et al., 2009; Xiu et al., 2016). Pantke et al. (2012) reported that green rust is a highly reactive iron mineral and

can be used for the recovery of chromium and arsenic. Furthermore, Fe(III) oxides can be recycled and used for phosphorus removal (Nielsen and Nielsen, 1998; Zhang et al., 2015a). Moreover, Fe(II)-driven denitrification leads to either encrusted or non-encrusted biominerals. Some studies reported that crust was formed around the cells during the autotrophic and mixotrophic growth (Kappler et al., 2005; Posth et al., 2014). Conversely, when supplementing chelating agents (i.e. EDTA) Fe(III) mineral encrustation is prevented (Chakraborty and Picardal, 2013).

The evaluation of the particular Fe(III) precipitates formed is crucial in order to decide the adoption of the “reuse strategy” and the possible applications. Some of the major applications are pigments for paints and the construction industry, magnetic pigments and ferrites, catalysts for industrial syntheses, adsorbents for water and gas purification and for low level radioactive waste decontamination, production of photochemicals and fertilizers (Cornell and Schwertmann, 2003).

The objectives of this work were to characterize the Fe(III) oxides by different mineral characterization methods, i.e. X-ray fluorescence (XRF), Raman spectroscopy, scanning electron microscopy equipped with energy dispersive X-Ray Analyser (SEM-EDX) and Fourier transformation infrared spectroscopy (FTIR).

5.2 Materials and methods

5.2.1 Sources of microorganisms and mineral growth media

Microorganisms and mineral media were as previously described (see sections 3.2.1 and 4.2.1).

5.2.2 Sampling and analytical methods

Mineral characterization of the Fe(III) precipitates formed during Fe(II)-mediated autotrophic denitrification was performed on: i) four samples obtained in the absence of metals, with the use of a *Thiobacillus*-dominated mixed culture (TMp), a *T. denitrificans* (TDPp), an activated sludge enrichment (ASp) and *Pseudogulbenkiania* strain 2002 (PGp); ii) twelve samples obtained in the experiments with Cu (TMCp, TDPCp, ASCp, PGCP), Ni (TMNp, TDPNp, ASNp, PGNp) and Zn (TMZp, TDPZp, ASZp, PGZp). Furthermore, the characterization of the precipitates in abiotic controls (ACp) was also performed.

Fe(III) oxides were pasteurized (80°C for 10 min), collected by centrifugation under anaerobic conditions, and washed twice with a NaHCO₃ buffer (pH 6.8) as previously described (Weber et al., 2001). Then, the precipitates were washed several times with double-distilled water by centrifugation, in order to remove the impurity ions associated with the procedure, as described in Cornell and Schwertmann (2003). Finally, the samples were dried at 40°C for 48 h prior to analysis.

The samples were firstly analyzed by using X-ray fluorescence (XRF) with a Niton XL3t GOLDD + XRF analyzer (Thermo Fisher Scientific, USA). The solid samples were also examined by scanning electron microscopy (SEM) in order to obtain the morphology information. The microscope was a Vega 3 (TESCAN, Czech Republic), equipped with an energy dispersive QUANTAX X-Ray Analyser (EDX) (Bruker, Czech Republic) for chemical analysis. Elemental identification and quantitative compositional analysis were performed by XRF and, in some cases, were supported by SEM-EDX. SEM microscopy was used to determine crystal size, morphology and domain character that could be directly observed.

FTIR spectra were recorded with an IRAffinity-1 Fourier transformation infrared spectrophotometer (Shimadzu, Japan), equipped with a deuterated-triglycine sulfate (DTGS) detector using a wavelength between 400 – 7000 cm⁻¹. Prior to being analyzed with FTIR, the samples were transformed into pellets (diameter 7 mm) by using potassium bromide (KBr). Infrared spectra were obtained through the interaction of iron oxides with electromagnetic radiation. FTIR was used to obtain a rapid and useful identification of Fe(III) precipitates and information about crystal morphology, degree of crystallinity and the extent of metal substitution (Gotic and Music, 2006). Furthermore, with FTIR spectroscopy, information about the adsorbed molecules and the nature of surface complexes can be obtained (Cornell and Schwertmann, 2003). Impurities that can produce distinct bands such as 1400 cm⁻¹ of nitrate and even traces of 1-2% of goethite in a sample of hematite can be identified by FTIR (Cornell and Schwertmann, 2003). Broadening of the absorption bands reflects a decrease in crystal perfection (Cambier, 1986). Structural substitution of other elements such as Al, Mn and Cr causes a shift in the positions of the bands (Cornell and Schwertmann, 2003).

Raman measurements were carried out on a Renishaw INVIA spectrometer equipped with a microscope and a CCD detector (LGE, France). A 532 nm solid-state green (Nd:YAG) laser was used with a maximum power of 50 mW. The acquisitions were performed through a Leica (x100) magnification objective after achieving a calibration on a silicon standard. The

Rayleigh scattering component was removed by an Edge filter, and the Raman-scattered light was dispersed by a holographic grating with 1800 lines/mm. The integration time was set at 60 s. Raman provided complementary information to that from infrared spectra (Cornell and Schwertmann, 2003) as Raman spectroscopy is an easy and fast method for the identification of different iron oxides, hydroxides and oxyhydroxides (Hanesch, 2009). This method can be used when a material is poorly crystallized which often occurs in bioprocesses (Hanesch, 2009). The spectra of the samples measured in this study were displayed and compared to those reported in the literature. An overview of the bands measured for various iron minerals was given by Cornell and Schwertmann (2003).

5.3 Results

Table 5.1 reports the FTIR and Raman bands that are defined as diagnostic for individual Fe(III) (hydr)oxides. However, in this study, a mixture of Fe(III)(hydr)oxides was identified in each sample. The different Fe(III) (hydr)oxides had similar FTIR and Raman spectroscopy bands. Furthermore, many impurities and different minerals apart from Fe(III) (hydr)oxides were present.

5.3.1 Color

An eye-catching color method was used for an easy and fast identification of the iron oxides (Cornell and Schwertmann, 2003). When the activated sludge enrichment was used, the liquid phase turned into reddish and brownish color at the end of the experiments. In the bottles with the *Thiobacillus*-mixed culture, the color was more red than brown. *T.denitrificans* and *Pseudogulbenkiania* strain 2002 resulted in a darker reddish brown or even black color. White, grey and reddish precipitates were observed in abiotic controls. In this study, the addition of Cu resulted in a red to brown liquid phase and a browner liquid phase was observed with the addition of Ni and Zn.

Table 5.1: Color and FTIR/Raman bands that are defined as diagnostic for individual minerals (Cornell and Schwertmann, 2003).

Formula/ Mineral name	Color	FTIR, bands (cm ⁻¹)	Raman spectroscopy bands (cm ⁻¹)
Fe ₂ O ₃ / Hematite	Red	345, 470, 540	226, 245, 292, 411, 479, 612
γ-Fe ₂ O ₃ / Maghemite	Red to brown	400, 450, 570, 590, 630	344, 381, 486-490, 670, 718
α-FeO(OH)/ Goethite	Brownish-reddish, yellow	797, 890	205, 247, 300, 386, 418, 481, 549
γ-FeO(OH)/ Lepidocrocite	Reddish yellow	753, 1026, 1161,	219, 252, 311, 349, 379, 528, 638
β-FeO(OH)/ Akaganeite	Brownish yellow	410, 670, 820, 1050, 1630	314, 380, 549, 722
FeO(OH)/ Ferrihydrite	Dark reddish brown	450, 650, 941, 3430, 3615	370, 510, 710
Fe ₃ O ₄ /Magnetite	black	400, 580-590	309, 532-540, 667

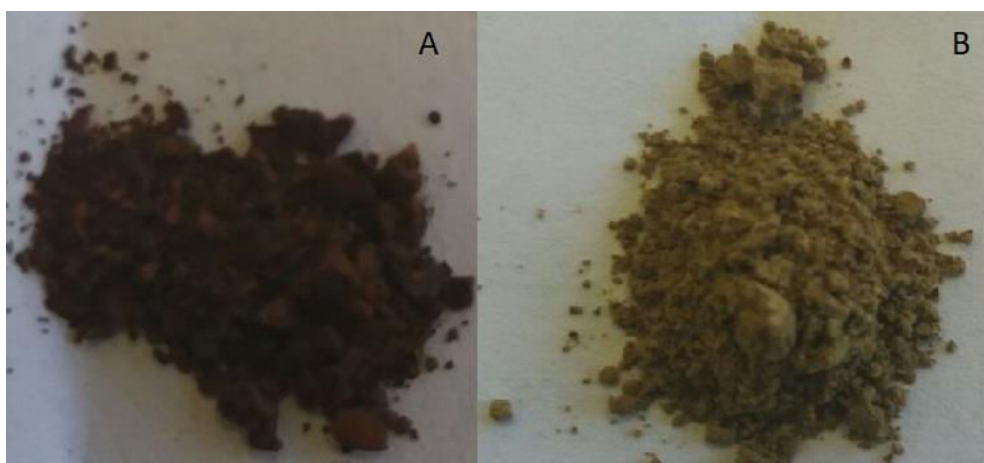


Figure 5.1: Samples obtained from abiotic controls (ACp) (A) and from *T. denitrificans* pure culture (TDPp) (B).

The color of Fe(III)(hydr)oxides observed by naked eye is reported in Table 5.1. However, Fe(III) precipitates consisted of a mixture of (hydr)oxides with many impurities and the color identification was thus not reliable. When transformed into a powder, the precipitates showed a different color, which is reported in Table 5.2. In Fig. 5.1, an example of the color of the ACp and TDPp precipitates is shown.

5.3.2 Mineralogy

5.3.2.1 FTIR and Raman spectroscopy for the samples formed in the absence of metals

FTIR and Raman spectra of the samples showed similar bands. Raman spectra of some characteristic bands are shown in Fig. 5.2. All spectra showed a band at around 1000 cm^{-1} , indicating the presence of siderite (iron carbonate mineral: FeCO_3) (Figure 5.2 b, c). Some spectra also showed a band at around $150\text{--}200\text{ cm}^{-1}$ (Fig. 5.2b). Figure 5.2a shows a typical 2-line ferrihydrite (Hanesch, 2009) of a dark spot in the sample PGNp.

The infrared spectrum of the precipitates in the abiotic controls (ACp) showed strong bands at 3380 , 1630 , 1049 and 540 cm^{-1} and a weak band at 470 cm^{-1} (Fig. 5.3). The bands at 540 , 470 and 3380 cm^{-1} are diagnostic for hematite. The FTIR band at 1630 and 1050 cm^{-1} was probably due to the formation of goethite or akaganeite. Dark and red spots (diameter $\sim 10\text{ }\mu\text{m}$) were observed with the microscope equipped in Raman spectroscopy instrument. The Raman spectra of the dark spots showed bands at 225 , $290\text{--}300$, 412 and 611 cm^{-1} that are diagnostic for hematite (Fig. 5.2b). Furthermore, because of the red spots, a band at 1321 cm^{-1} was also observed, corresponding to hematite. No goethite or akaganeite bands were observed in Raman spectra.

The infrared spectrum of the precipitates with *Thiobacillus-mixed* culture showed the same bands of those obtained in the abiotic controls, with the only exception that the band of hematite at 470 cm^{-1} was not observed. Furthermore, strong bands at 1630 and 1050 cm^{-1} most likely corresponded to akaganeite, as confirmed by the two broad bands at 700 and ca. 400 cm^{-1} obtained for the red spots in Raman spectra (diameter $3\text{ }\mu\text{m}$). Dark and red spots of TMp were smaller than in abiotic controls (Fig.5.4). The dark spots (with a diameter of approximately $2.5\text{ }\mu\text{m}$) corresponded to hematite as the combination of bands 225 , $290\text{--}300$ and 412 cm^{-1} was observed. Additional strong bands at 120 and 1050 cm^{-1} were likely due to the presence of impurities. Another possibility is that the band might have been at around 700 cm^{-1} , due to ferrihydrite. Furthermore, some more white spots were detected in the sample. The bands were similar to the bands of the red spots, however the band at 400 cm^{-1} was more intense.

The same FTIR bands were observed when the *T. denitrificans* pure culture (TDPp) was used. Similarly, Raman spectra of the dark spots (approximately diameter $5\text{ }\mu\text{m}$) showed bands at 225 , $290\text{--}300$, 412 cm^{-1} and, additionally, at 611 cm^{-1} , with all the bands corresponding to hematite. However, Raman spectra of the red spots (diameter $\sim 5\text{ }\mu\text{m}$)

identified bands at 700 and 1600 cm^{-1} that possibly corresponded to maghemite. However, the band at 700 cm^{-1} could also represent two-line ferrihydrite. The additional bands at 1400 and 2360 cm^{-1} were impurities.

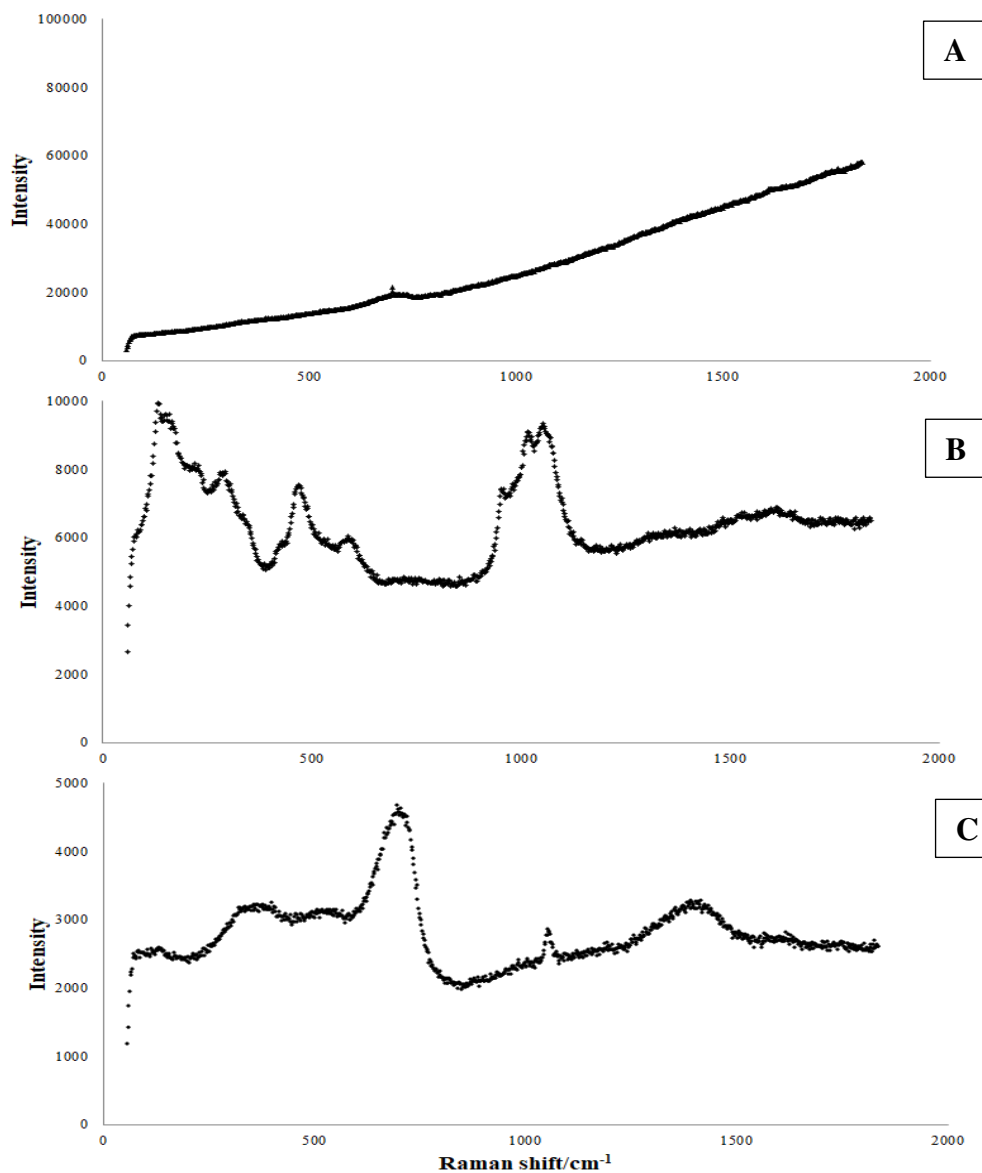


Figure 5.2: Raman spectra of the samples obtained during the experiments with A) *Pseudogulbenkiania* strain 2002 in the presence of Ni (PGNp) (dark spot), B) *Pseudogulbenkiania* strain 2002 (PGp) (dark spot), C) activated sludge in the presence of Cu (ASCp) (white spot)

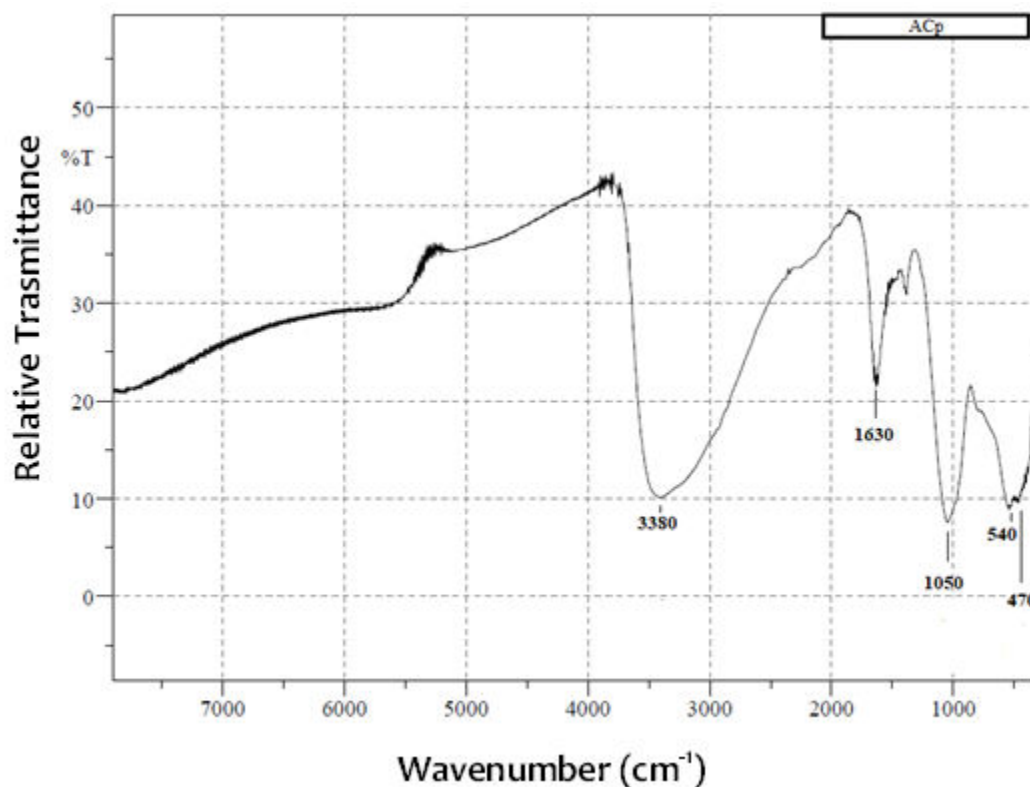


Figure 5.3: FTIR spectra of the precipitate obtained during the abiotic controls

The FTIR spectrum of ASp samples collected from activated sludge bottles showed bands at 540, 1050, 1630 and 3380 cm^{-1} , as the previous samples that corresponded to hematite. The Raman spectra of dark spots confirmed the presence of hematite. Additionally, bands at 1300 and 1600 cm^{-1} in dark and red spots might correspond to maghemite and/or magnetite. The Raman spectrum of white spots showed bands at 350, 500, 700 and 1300 cm^{-1} . However, the bands at 3740 and 3725 cm^{-1} , corresponding to OH groups of maghemite were not recorded. Most possibly, the spectrum was therefore a mixture of magnetite and maghemite. The broad band from 653 to 750 cm^{-1} was caused by both minerals. The maghemite band above 1300 cm^{-1} was relatively weak compared to the other bands, probably due to the presence of magnetite. However, the red spots might also have contained six-line or two-line ferrihydrite, corresponding to the three bands observed at 350, 500 and 700 cm^{-1} .

Similarly, sample PGp from the bottles inoculated with *Pseudogulbenkiania* strain 2002 showed the same FTIR bands of hematite, such as the Raman spectra of the dark spots.

As in previous samples, an additional band at 1600 cm^{-1} might indicate the presence of maghemite.

Table 5.2: Color, FTIR and Raman spectroscopy bands of the samples

Samples	Color	FTIR, bands (cm^{-1})	Raman spectroscopy bands (cm^{-1})	Minerals
ACp	Reddish	470, 540, 1050, 1630, 3380	Dark/Red: 225, 290-300, 412, 470, 611, 1321	Hematite (Goethite or Akaganeite)
TMp	Red to brown	540, 1050, 1400, 1630, 3380	Dark: 225, 290-300, 412 Red: 400, 700	Hematite and Akaganeite (and/or Ferrihydrite)
TDPp	Brownish-reddish, yellow	540, 1050, 1400, 1630, 3380	Dark: 225, 290-300, 412, 611 Red: 700, 1600	Hematite and Maghemite and/or 2-line Ferrihydrite
ASp	Dark reddish brown	540, 1050, 1630, 3380	Dark1: 225, 290-300, 412, 611 Dark2: 225, 290-300, 412, 700, 1300, 1600 Red: 400, 700, 1300, 1600 White: 400, 500, 700, 1300	Hematite, Maghemite and/or magnetite (and/or Ferrihydrite)
PGp	Brownish-reddish, yellow	540, 1050, 1630, 3380	Dark: 225, 290-300, 470, 1600	Hematite and maghemite
TMCp	Red to brown (yellow)	470, 500, 540, 1050, 1400, 1630, 3448	Dark: 225, 290-300, 412 Red: 400, 700	Hematite and Akaganeite
TDPCp	Brownish-reddish	470, 1050, 1400, 1630, 3380	Dark/red: 225, 400, 700, 1300	Hematite and Maghemite and/or 2-line Ferrihydrite
ASCp	Brownish-reddish	470, 500, 1050, 1400, 1630, 3448	Dark1: 225, 290-300, 412, 611 Dark2: 225, 290-300, 412, 700, 1300, 1600 Red: 400, 700, 1300, 1600 White: 400, 500, 700, 1300	Hematite, Maghemite and/or magnetite (and/or Ferrihydrite)
PGCp	Black	470, 1050, 1400, 1630, 3380	Dark: 400, 700, 1300	Hematite and maghemite
TMNp	Red to brown	450, 650, 940, 1630, 3430	Dark, red: 400, 700 1321	Hematite Akaganeite/Ferrihydrite, and (possible Maghemite)
TDPNp	Red to brown	450, 1050, 1400, 1630, 3380	Dark, red: 400, 700 1321 Dark: Additional 1600	Hematite and Maghemite and/or 2-line Ferrihydrite
ASNp	Red to brown	450, 1050, 1400, 1630, 3380	White: 400, 700, 1321, 1600	Hematite, Maghemite, (and/or Ferrihydrite)
PGNp	Red to brown	450, 1050, 1400, 1630, 3430	Dark, red: 400, 700 1321	Hematite and maghemite
TMZp	Red to brown	410, 940, 1630, 3440	Dark, red: 225, 245, 411, 700, 1300	Hematite Akaganeite/Ferrihydrite, and (possible Maghemite)
TDPZp	Red to brown	450, 1050, 1400, 1630, 3380	Dark, red: 400, 700 1321 Red/White: Additional 1600 (Maghemite)	Akaganeite and Hematite (possible Maghemite)
ASZp	Red to brown	450, 940, 1050, 1400, 1630, 3380	Dark, red: 400, 700 1321 Red/White: Additional 1600 (Maghemite)	Hematite, Maghemite and/or magnetite (and/or Ferrihydrite)
PGZp	Black	450, 940, 1050, 1400, 1630, 3380	Dark, red: 225, 290-300, 412, 611, 700, 1321 Red/White: Additional 1600	Hematite and maghemite

5.3.2.2 FTIR and Raman spectroscopy of the precipitates formed in the presence of metals

Addition of Cu

The infrared spectrum of the precipitates obtained with the *Thiobacillus*-mixed culture in the presence of Cu (TMCp) showed the bands at 1050 cm^{-1} and 1630 cm^{-1} , corresponding to akaganeite. The addition of Cu resulted in a broad band at 3448 cm^{-1} , likely corresponded to the $3480+3390$ (doublet) (OH and H_2O stretch) of akaganeite, as also confirmed by the additional band at around 500 cm^{-1} that was due to the Fe-O stretch of akaganeite. The weak bands at 470 and 540 cm^{-1} likely corresponded to hematite. Besides, a strong band at around 1400 cm^{-1} corresponded to nitrate. Raman spectrum of the dark, red and white spots showed the same bands as in the Tmp precipitates. TMCp was most likely a mixture of hematite and akaganeite as Tmp.

As observed using the *Thiobacillus*-mixed culture, the mineralogy of the precipitates was not considerably affected by the presence of Cu when the *T. denitrificans* pure culture was used. The infrared spectrum showed the same bands of the TDPp sample, despite another band of hematite at 470 instead of 540 cm^{-1} was recorded in the TDPCp sample. The Raman bands at 225 , 412 and 1321 cm^{-1} corresponded to hematite and additional band at 700 cm^{-1} probably indicated the presence of two-line ferrihydrite. The Raman spectra of TDPp samples showed band of maghemite at 1600 cm^{-1} , which, however, was not identified with the addition of Cu. The bands at 700 and 1330 cm^{-1} were likely due to maghemite, in agreement with what was observed in sample TDPp.

Similarly, ASCp sample collected from activated sludge bottles in the presence of Cu in the liquid phase showed the same FTIR bands as for TMCp. Hematite, maghemite and/or magnetite and/or two-line ferrihydrite were identified from the Raman spectra. PGCp sample confirmed that the addition of Cu resulted in same Fe(III) (hydr)oxides of the precipitates in the absence of metals.

Addition of Ni

Infrared spectrum obtained from the sample of the bottles inoculated with *Thiobacillus*-mixed culture in the presence of Ni (TMNp) showed some additional FTIR peaks at 3430 , 940 , 650 and 450 cm^{-1} , corresponding to ferrihydrite. As in previous samples, a strong band at 1630 cm^{-1} , might correspond to akaganeite; however, the band at 1050 cm^{-1}

was not observed. In contrast, none of the FTIR bands of hematite were recorded in TMNp sample. The Raman spectrum of the dark spots showed the same bands similarly to previous samples, corresponding most possibly to hematite. Red spots might be either akaganeite or ferrihydrite as bands at about 400 and 700 cm^{-1} were recorded. However, the additional broad band of either maghemite or hematite at about 1300 cm^{-1} was recorded. Similarly, the TDPNp, ASNp and PGNp samples showed the bands reported in Table 5.2 and described in section 5.3.2.2.

Addition of Zn

FTIR spectrum of the sample that was taken from bottles inoculated with *Thiobacillus*-mixed culture in the presence of Zn (TMZ) showed a mixture of ferrihydrite and akaganeite. The FTIR band at 3430 cm^{-1} and the broad band at 940 cm^{-1} of ferrihydrite were observed. The bands at 1630 cm^{-1} , 410 and 1050 cm^{-1} corresponding to akaganeite were recorded. Furthermore, an additional band at 1384 cm^{-1} was observed. Hematite among the FTIR bands was not confirmed. Dark, red and orange spots were observed by Raman spectroscopy, however as in TMNp sample, the dark spots had similar peaks with the red spots. As described above, red spots could be akaganeite or ferrihydrite or a mixture of them, in agreement with the FTIR analysis results. In addition, in this sample, orange spots were observed, which might correspond to hematite, as hematite peaks at 225, 245, 411 and 1321 cm^{-1} were visible. The bands of TDPZp, ASZp and PGZp are reported in Table 5.2.

5.3.3 Elemental identification and quantitative compositional analysis by XRF and SEM-EDX

The precipitates obtained in the experiments with Cu, Ni, Zn indicated absorption and/or co-precipitation with Fe(III)(hydr)oxides. Table 5.3 reports Fe oxides amount (%) in each sample. The use of *Thiobacillus*-mixed culture and chemical oxidation resulted in 61% of Fe(III) oxides in the sample. A slightly lower percentage of 57 and 55% was observed when the *T. denitrificans* mixed culture and the activated sludge enrichment were used, respectively. The use of *Pseudogulbenkiania* strain 2002 resulted in 50% of Fe(III) oxides in the precipitate. When Cu was supplemented, a higher amount of Fe(III) oxides by 20 and 15% was obtained with the *T. denitrificans* and *Pseudogulbenkiania* strain 2002 pure cultures, respectively, whereas a decrease by 14 and 13% was observed in the samples with

the *Thiobacillus*-mixed culture and activated sludge enrichment. The addition of Ni resulted in an increase of the Fe(III) oxides formation in the range of 5-20% in all the samples. The precipitates in the bottles with Zn had a lower amount of Fe oxides by 6 and 2% with the *Thiobacillus*-mixed culture and the *Pseudogulbenkiania* strain 2002, respectively, whereas an increase by 22 and 9% was observed in bottles with activated sludge and the *T. denitrificans* pure culture.

Table 5.3: Elemental identification and quantitative compositional analysis by XRF and SEM-EDX

Samples	Total Fe oxides in the sample	Elemental composition (%)													
	Fe ₂ O ₃ (%)	Fe	P	Cl	K	Na	Mg	Si	Al	Ca	Mn	S	Cu	Ni	Zn
ACp	61	54.40	8.14	1.27	0.56	1.40	0.19	0.14	0.13	0.13	0.12				
TMp	61	56.69	10.97	0.69	0.51	1.60	0.43	0.15	0.05	0.25	0.05	0.15			
TDPp	57	55.82	10.19	0.42	0.81	1.22	0.20	0.00		0.10	0.05				
ASp	55	53.21	9.53	2.04	0.78	1.60	0.69	0.81		1.48	0.12				
PGp	50	43.28	7.61	4.39	0.76	2.20		0.15		0.20	0.05				
TMCp	47	41.16	8.09	4.95	1.09	5.66	0.25	1.17		0.19	0.14		0.40		
TDPCp	77	70.79	1.31	1.61	0.10	0.00	0.42	1.97		0.10	0.08		0.78		
ASCp	44	40.69	1.82	7.52	1.32	9.14	0.40			0.16	0.04		0.45		
PGCp	65	58.14	0.80	1.43	0.10	1.10	0.30	1.00		0.08	0.11		0.54		
TMNp	81	76.84	1.16	2.03	0.22	1.30	0.20	1.00		0.00				0.31	
TDPNp	77	69.68	2.92	0.78	0.29	4.57	1.05			0.04	0.11			0.28	
ASNp	73	66.21	0.86	0.71	0.09	0.00		1.30	0.10	0.08	0.10			0.25	
PGNp	55	47.48	0.62	5.27	0.68	7.26		1.43		0.15				0.35	
TMZp	54	50.10	2.39	7.61	0.61	5.00	0.44	0.08		0.00		0.15			0.20
TDPZp	66	55.75	3.10	1.32	0.20	3.20	0.30	1.22		0.12	0.11				0.23
ASZp	77	66.68	1.50	1.54	0.15	5.02	0.30	1.23		0.07	0.13				0.19
PGZp	48	37.42	0.49	1.88	0.00			1.01							0.18

5.3.4. Microscopy

Samples were observed with the microscope equipped in Raman spectroscopy instrument. None of the samples was homogeneous. A mixture of several spots (dark, white, red and orange) in microscale was observed (Fig. 5.4). As the spot sizes were mostly small in a variety in the range 100 nm - 10 µm, several spots on each sample were measured to avoid the measurement of a small impurity instead of Fe(III) (hydr)oxides. Two samples, i.e. TMp and TDPp, were inspected by SEM, which revealed varying sizes and geometrical shapes. Fig. 5.5 shows the SEM micrographs of sample TMp (three details, a, b, c) and sample TDPp (three details, d, e, f). All previous analyses have confirmed that these samples contained a mixture of Fe(III) (hydr)oxides in different sizes. Fig. 5.5 a, b, c shows that the particles of

TMp were not aggregates but individuals in a variety of sizes and shapes with a diameter ranging from about 1 micrometer to 1 mm. Similarly, Fig. 5.5 d, e, f shows a higher amount of smaller particles with a diameter in the range from 1 μm to 0.5 mm, than those observed in the TMp sample. Rod, modular and irregular shape particles were observed in TMp, whereas more spherical shape was identified in TDPp.

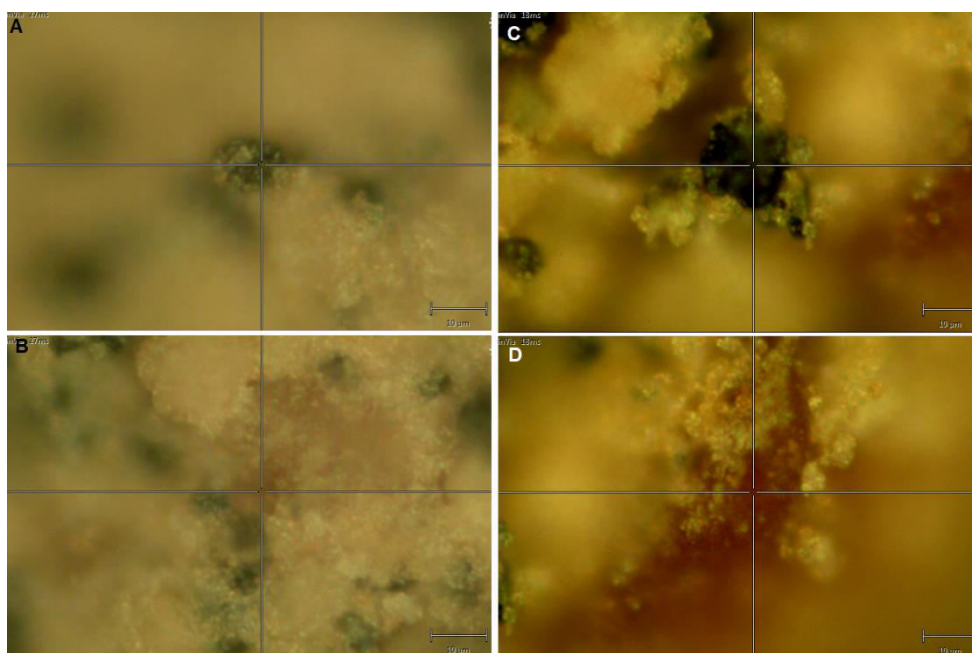


Figure 5.4: Raman microscopy of the dark and red spots in the precipitates A,B) from bottles inoculated with *Thiobacillus*-mixed culture (TMp) (A, B) and C, D) from abiotic controls

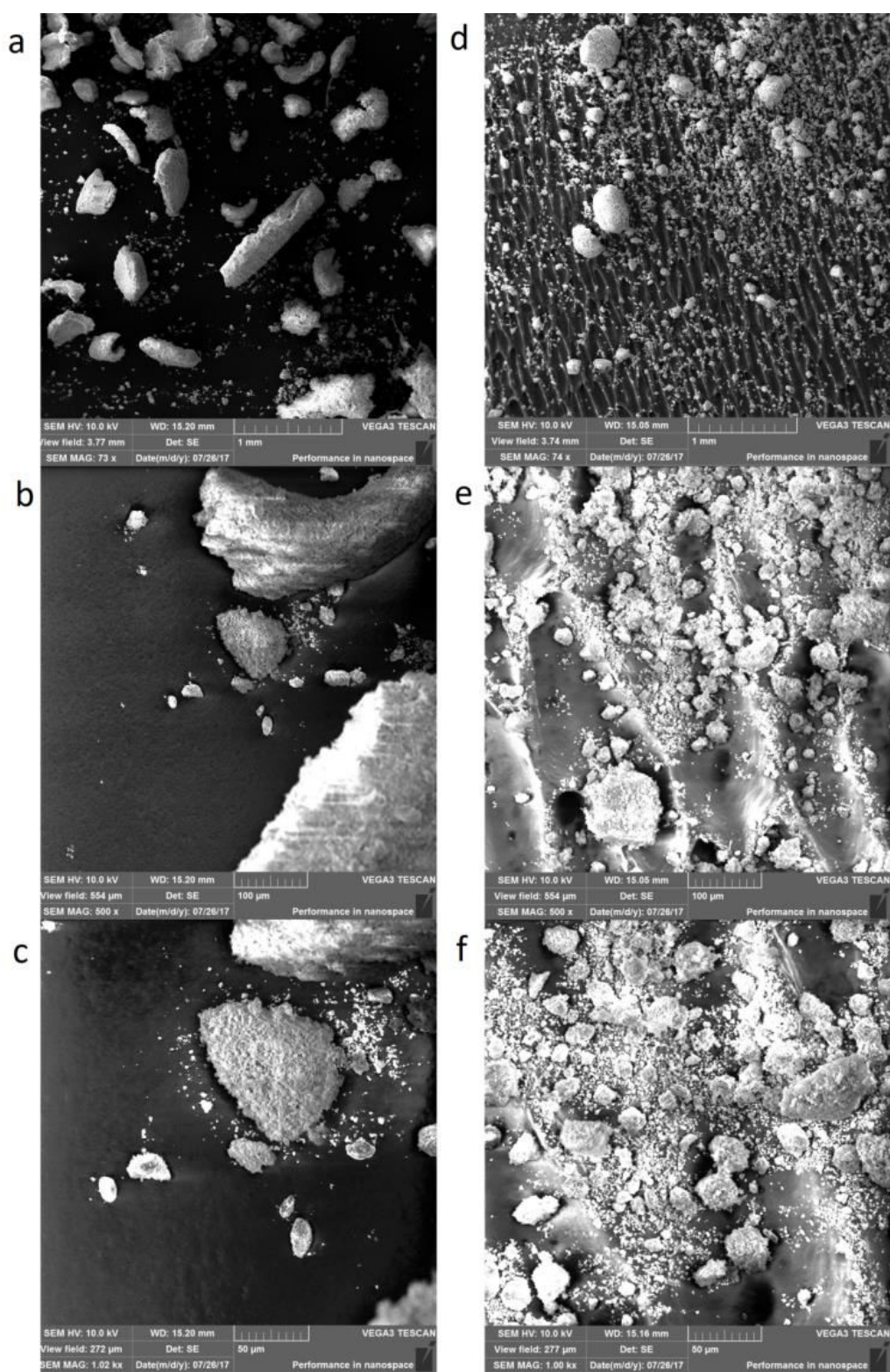


Figure 5.5: SEM micrographs of samples were obtained from bottles inoculated with *Thiobacillus* mixed culture (three details (a, b, c)) and sample *T. denitrificans* pure culture (three details (d, e, f))

5.4 Discussion

5.4.1 Comparison between the precipitates formed during chemical oxidation and Fe(II) mediated autotrophic denitrification

Iron oxides are valuable minerals for many applications due to their low cost abundance and physical-chemical properties (Pouran et al., 2014; dos Santos et al, 2016). Among Fe(III) (hydr)oxides, hematite, maghemite, magnetite and goethite have the greatest interest in industry (Cao et al, 2015).

FTIR and Raman spectroscopy of the samples formed during Fe(II) mediated autotrophic denitrification showed a mixture of Fe(III) (hydr)oxides in each sample. The bands observed were similar, indicating that the medium composition and the incubation conditions are important factors for the Fe(III) (hydr)oxides formation. However, the differences between the precipitates obtained in the abiotic controls and the experiments with the microorganisms showed that the different cultures resulted in diverse biogenic Fe(III) (oxyhydr)oxides minerals, in agreement with other studies (Senko et al., 2005a, b; Posth et al., 2014). Furthermore, the differences in the color of the liquid phase indicated that the different microbial cultures likely affected the mineralogy of Fe(III) (hydr)oxides.

All spectra showed a band at around 1000 cm^{-1} , indicating the presence of siderite. Siderite spectra consisted of bands at 184, 287, 731 and 1090 cm^{-1} (Rull et al., 2004), with the most common band being at 1090 cm^{-1} . Amounts of phosphate and carbonate minerals were recorded by XRF. Similarly, the use of the *Acidovorax* strain BoFeN1 in the presence of Fe(II) and acetate resulted in a mixture of Fe(III) (hydr)oxides, vivianite and amorphous Fe(III)-phosphate in the studies by Miot et al. (2009) and Schaedler et al. (2009).

Ferrihydrite was most probably contained in all the samples, in agreement with the majority of studies on nitrate-dependent ferrous oxidation (Straub et al., 1996, 2004; Hafenbradl et al., 1996; Lack et al., 2002; Senko et al., 2005 a, b; Hohmann et al., 2009; Chakraborty et al., 2011; Zhao et al., 2013; Miot et al., 2014; Li et al., 2014, 2015). However, FTIR was not a suitable method for the identification of ferrihydrite precipitates that are impure and poorly crystalized (2-line ferrihydrite). The Mössbauer spectroscopy might have supported a better characterization of such precipitates (Cornell and Schwertmann, 2003). According to Emerson and Moyer (1997), the solids that were formed by microaerophilic oxygen-dependent Fe(II)-oxidizing bacteria were poorly crystalline Fe(III) precipitates, such as ferrihydrite. However, poorly crystalline phases in aqueous solution have been reported to

transform into goethite, hematite, lepidocrocite and other crystalline forms depending on the pH and solution chemistry (Schwertmann and Cornell, 2000; Kappler and Newman, 2003). This stands in line with this study, with ferrihydrite that was always present with other more crystalline phases. Other studies reported that in abiotic systems the transformation of the initial precipitates into more crystalline phases was slower than that observed with bacteria. This was due to the presence of reducing agents or Fe(II) in combination with surface bridging ligands that are known for stimulating reductive dissolution of poorly crystalline Fe(III)-oxides and subsequent re-precipitation of more highly ordered phases (Cornell et al., 1989; Cornell et al., 1991; Cornell and Schwertmann, 2003; Kappler and Newman, 2003). In this study, however, the mineralogy of the precipitates was only analyzed at the end of the experiments, and no additional information can be given on the production rate of the minerals in the presence or absence of microorganisms.

In this study, white, grey and reddish precipitates were observed in abiotic controls. In agreement, white precipitates from chemical Fe(II) oxidation in both biotic and abiotic experiments were reported elsewhere (Miot et al., 2009). Other studies also observed a white fluffy structure that turned into a greenish-gray or reddish/orange substance within a week after the start of the incubation (Chaudhuri et al., 2001; Miot et al., 2009; Pantke et al., 2012). Green rusts as major products cause the grayish-green appearance (Straub et al., 1996) and Fe(III) oxyhydroxide or ferrihydrite generally give an orange/brown color to the liquid phase (Lack et al., 2002).

Biogenic minerals showed smaller particles than those formed under abiotic conditions. SEM inspection showed varying sizes and geometrical shapes such as irregular, rod, modular and spherical in diameter ranging from about 1 μm to 1 mm (Fig. 5.5). Similarly, Kappler and Newman (2003) found that Fe(III) precipitates from bottles inoculated with the *R. ferrooxidans* strain SW2, showed a variety of particles in the range of hundreds of nm to 20 μm . These particles showed various shapes, e.g., needles and plates, as well as irregular shapes, as also confirmed by Kappler et al. (2005).

In this study, hematite ($\alpha\text{-Fe}_2\text{O}_3$), maghemite (Fe_2O_3) and akaganeite ($\beta\text{-FeOOH}$) were formed. In agreement, these Fe(III) (hydr)oxides can be formed in the presence of chloride ions (Raming et al., 2002; dos Santos et al., 2016) and metals provided as chloride salts. However, many impurities and different minerals apart from Fe(III) (hydr)oxides were present in all precipitates.

5.4.1.1 Precipitates in abiotic controls

Chemical Fe(II) oxidation mainly resulted in the formation of hematite and another mineral which can be goethite or akaganeite. The IR bands at 540 and 470 cm^{-1} are diagnostic for hematite (Cornell and Schwertmann, 2003), as also confirmed by the reddish color of the ACp sample. Furthermore, the strong band observed at 3380 cm^{-1} in the spectrum most likely corresponded to the hydroxyl groups in the hematite crystals (Rochester and Topham, 1979). The FTIR band at 1630 cm^{-1} was probably related to the position of H₂O bending vibrations of goethite, whereas the band at 1050 cm^{-1} could be inferred that the sample contained a significant amount of specifically adsorbed impurities in goethite particles (Gotic and Music, 2007). Park et al. (2014) also observed that chemical Fe(II) oxidation resulted in the formation of goethite.

The revealing of the band at 1630 cm^{-1} can be also related to the presence of the typical O-H bending of akaganeite (Cornell and Schwertmann, 2003). This derives from the additional band that was observed at 1050 cm^{-1} that has been reported in akaganeite samples in KBr discs, and considered to be artefacts (Murad and Bishop, 2000). The Raman spectra of the dark spots showed bands at 225, 290-300, 412 and 611 cm^{-1} that are diagnostic for hematite as reported in the FTIR analysis. Furthermore, because of the red spots, a band at 1321 cm^{-1} was also observed, corresponding to hematite. According to Oh et al. (1998), the bands of a well-established hematite have been shown at 225, 245, 291, 411, 500, 611 and 1321 cm^{-1} . No goethite or akaganeite bands were, however, observed in Raman spectra.

5.4.1.2 Mineralogy of the precipitates using nitrate-dependent ferrous oxidizers

Precipitates of the experiments carried out with the *Thiobacillus*-dominated mixed culture were a mixture of hematite, akaganeite and/or ferrihydrite. In industry, hematite is the most used Fe(III) (hydr)oxide, especially for its application as a pigment. Akaganeite has electrochemical properties, high catalytic activity and good adsorption ability (Zhang et al. 2015b).

The strong band at 1630 and 1050 cm^{-1} most likely corresponded to akaganeite, in agreement with Raman spectra. The broad bands of akaganeite at 700 and ca. 400 cm^{-1} were obtained for the red spots (Boucherit et al., 1989). However, the band at around 700 cm^{-1} might have been due to ferrihydrite. In six-line ferrihydrite three bands at 370, 510 and 710 cm^{-1} and in two-line ferrihydrite only the strong 710 cm^{-1} band can be seen (Mazzetti and

Thistlethwaite, 2002). Six-line ferrihydrite is a “well”-crystalline ferrihydrite and two-line ferrihydrite is the most poorly crystalline form (Cornell and Schwertmann, 2003). However, in the infrared spectrum none of the FTIR bands of ferrihydrite at 3615, 3430, 650, 450 cm^{-1} was observed, indicating that the red spots probably corresponded to akaganeite. Furthermore, some more white spots were detected in the sample. The bands were similar to the bands of the red spots, however the band at 400 cm^{-1} was more intense, indicating the higher amount of most possible akaganeite.

When the pure culture of *T. denitrificans* was used, on top of hematite and/or ferrihydrite, maghemite was observed. Maghemite can be used as a magnetic pigment in electronics, due to its low cost and chemical stability. Furthermore, maghemite nanoparticles can be used in biomedicine (Dronskowski, R., 2001; Pankhurst et al., 2003). The FTIR and Raman analyses confirmed the presence of hematite. Raman spectra of the red spots identified bands at 700 and 1600 cm^{-1} that possibly corresponded to maghemite (Hanesch, 2009). No other maghemite bands at 350, 512, 664, 726 and 1330 cm^{-1} were identified, in contrast to what reported by Jacintho et al. (2007). However, the band at 700 cm^{-1} could also represent two-line ferrihydrite.

The activated sludge enrichment allowed the production of hematite and maghemite and/or magnetite (and/or ferrihydrite). The FTIR spectrum showed bands as the previous samples and Raman spectra confirmed the presence of hematite. Additionally, bands at 1300 and 1600 cm^{-1} in dark and red spots might correspond to maghemite and/or magnetite. The Raman spectrum of white spots showed bands at 350, 500, 700 and 1300 cm^{-1} , very similar to the maghemite bands at 350, 512, 664, 726 and 1330 cm^{-1} reported by Hanesch (2009). However, the bands at 3740 and 3725 cm^{-1} , corresponding to OH groups of maghemite were not recorded (Busca et al., 1993). De Faria et al. (1997) reported three broad peaks at 350, 500 and 700 cm^{-1} in maghemite spectrum. According to Hanesch (2009), the broad band from 673 to 722 cm^{-1} that can be observed in all samples can be caused by magnetite and maghemite. Magnetite bands were observed at 309 and 540 cm^{-1} , whereas maghemite peaks at 344 and 490 cm^{-1} . Most possibly, the spectrum was therefore a mixture of magnetite and maghemite. In agreement Hanesch (2009), studied the biomineralized material produced in the soil by anaerobic iron-reducing bacteria and found a mixture of Fe(III)(hydr)oxides that consisted of magnetite and maghemite. The broad band from 653 to 750 cm^{-1} was caused by both minerals. The maghemite band above 1300 cm^{-1} was relatively weak compared to the other bands, probably due to the presence of magnetite. However, the red spots might also

have contained six-line or two-line ferrihydrite, corresponding to the three bands observed at 350, 500 and 700 cm⁻¹.

The use of the pure culture of *Pseudogulbenkiania* strain 2002 resulted in hematite and maghemite formation (and/or ferrihydrite). FTIR spectra showed the bands of hematite, such as the Raman spectra with the additional band at 1600 cm⁻¹ corresponding to maghemite. Zhao et al. (2013) reported that the minerals that were formed with the use of *Pseudogulbenkiania* strain 2002 were nontronite, a clay mineral, but also other secondary minerals such as vivianite, ferrihydrite and magnetite. Xiu et al. (2016) used *Pseudogulbenkiania* strain 2002 and reported that lepidocrocite was the only crystalline biogenic solid formed. However, in this study, the medium was different as acetate and arsenic, which affected the crystallinity of the precipitates, were not added.

In agreement to this study, Sun et al. (2009) reported the formation of a mixture of Fe(III) oxides dominated by crystalline hematite with the use of an anaerobic sludge inoculum from a lab-scale As(III)-oxidizing denitrifying bioreactor. In other studies with nitrate-dependent Fe(II) oxidizers, biogenic goethite formation was reported by the use of *Acidovorax* strain BoFeN1 using Fe(II) and acetate as co-electron donor (Kappler et al., 2005; Hohmann et al., 2009; Larese-Casanova et al., 2010; Pantke et al., 2012; Klueglein and Kappler, 2013; Miot et al., 2014). Larese-Casanova et al. (2010) demonstrated that goethite was produced by abiotic Fe(II) oxidation in the presence of high concentrations of HCO₃⁻. Goethite was also reported with the use *Klebsiella* Strain FW33AN a nitrate-dependent Fe(II)-oxidizing bacterium (Senko et al., 2005a, b). The formation of amorphous or less crystalline Fe(III) oxide minerals by the use of *Sphaerotilus natans* strain DSM 6575^T in mixotrophic conditions with pyruvate and Fe(II) was reported (Park et al., 2014).

5.4.2 Mineralogy for the precipitates in the presence of metals

Nickel, copper and zinc have been reported to be essential for the structure of several precipitates, such as akaganeite and maghemite (Deliyanni et al. 2007; Drofenik et al., 2008; Nador et al, 2013; dos Santos et al., 2016; Hu et al., 2016; Suzuki et al., 2017). However, no previous studies have investigated the effects of these metals on the formation of biogenic minerals by nitrate-dependent Fe(II)-oxidizing microorganisms. In this study, all the precipitates contained Cu, Ni, Zn, indicating the absorption and/or co-precipitation with

Fe(III)(hydr)oxides. This is in agreement with the decrease of the soluble Cu, Ni and Zn concentration that was observed in the batch experiments (chapter 4).

The addition of Cu, Ni and Zn resulted in similar Fe(III) (hydr)oxides of the precipitates obtained in the absence of metals. However, the infrared spectrum of the precipitate obtained with the *Thiobacillus*-mixed culture in the presence of Cu (TMCp) showed an additional band at around 500 cm^{-1} that was due to the Fe-O stretch of akaganeite. This additional band might be due to a higher amount of akaganeite formed because of Cu. Another explanation is that the strong band of 3448 cm^{-1} might be attributed to the surface hydroxyl groups of goethite, but the intense band of hydroxyl stretch of goethite at 3140 cm^{-1} (Cornell and Schwertmann, 2003) and the typical band at 3666 cm^{-1} were not observed, most likely due to the presence of phosphate in the medium as reported by Russell et al. (1979). TMCp was most likely a mixture of hematite and akaganeite as TMp, indicating that the addition of Cu did not affect the mineralogy. Similarly, using the other microbial cultures, the mineralogy of the precipitates was not considerably affected by the presence of Cu. However, the addition of Ni and Zn likely stimulated the formation of maghemite.

The infrared spectrum obtained from the sample of the bottles inoculated with *Thiobacillus*-mixed culture in the presence of Ni (TMNp) showed some additional peaks. One band was revealed at 940 cm^{-1} , which has been reported when ferrihydrite contains a few percent of Si (Schwertmann and Thalmann, 1976). In this study, the presence of Si was confirmed by the XRF analysis. Additionally, the band at 450 cm^{-1} and a weak band at 650 cm^{-1} might correspond to bulk OH deformations of ferrihydrite, whereas the band at 3430 cm^{-1} can be explained as the bulk OH stretch of ferrihydrite. As in previous samples, a strong band at 1630 cm^{-1} likely indicated the presence of akaganeite. However, the band at 1050 cm^{-1} was not observed. None of the typical FTIR bands of hematite was recorded in TMNp sample, but hematite was revealed by the Raman spectrum of the dark spots. Red spots might be either akaganeite or ferrihydrite as bands at about 400 and 700 cm^{-1} were recorded. The additional broad band of either maghemite or hematite at about 1300 cm^{-1} was observed, indicating that the addition of Ni, might stimulate the formation of maghemite. Similarly, the same Fe(III) (hydr)oxides of the precipitates as in the absence of metals and the stimulation of the formation of maghemite was observed in TDPNp, ASNp and PGNp samples.

The FTIR spectrum of the precipitates taken from the bottles inoculated with *Thiobacillus*-mixed culture in the presence of Zn (TMZ) showed a mixture of ferrihydrite and akaganeite. An additional band at 1384 cm^{-1} might be associated with nitrate in ferrihydrite

(Cornell and Schwertmann, 2003). The FTIR and Raman spectra were similar to those obtained with the addition of Ni for all microbial cultures, indicating that also the presence of Zn likely stimulated the formation of maghemite.

5.5 Conclusions

Fe(II)-mediated autotrophic denitrification resulted in the formation of a mixture of Fe(III) (hydr)oxides of poorly crystalline (e.g. ferrihydrite), but also in some crystalline phases such as hematite, akaganeite and maghemite. In abiotic controls, the chemical Fe(II) oxidation resulted in hematite formation and goethite or akaganeite. Precipitates of the experiments carried out with the *Thiobacillus*-dominated mixed culture were a mixture of hematite, akaganeite and/or ferrihydrite. When *T. denitrificans* was used on top of hematite and/or ferrihydrite, maghemite was observed. The use of pure culture of *Pseudogulbenkiania* strain 2002 resulted in hematite and maghemite formation and the activated sludge enrichment allowed the production of hematite and maghemite and/or magnetite (and/or ferrihydrite). No difference in the mineralogy of the precipitates was observed with the addition of Cu, whereas the addition of Ni and Zn markedly stimulated the formation of maghemite. Heavy metals in the precipitates were identified by chemical analysis, indicating co-precipitation and/or absorption in Fe(III) (hydr)oxides. The recovery of heavy metals is an important application for Fe(II)-mediated autotrophic denitrification.

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CHAPTER 6

Evaluation of Fe(II)-mediated autotrophic denitrification in continuous flow packed-bed reactors

6.1 Introduction

Nitrate contamination of water resources is an increasingly serious global problem which has a significant impact on environment, such as eutrophication, and human health, i.e cancer and methemoglobinemia (Zhang et al., 2015). Nitrate pollution is mainly due to the use of chemical fertilizers and the discharge of industrial, domestic and nitrate containing wastewaters, such as landfill leachate (Manconi et al., 2007; Ghafari et al., 2008; Chen et al., 2014).

Physicochemical and biological denitrification methods are the main technologies for the removal of nitrate from water resources. Conventional methods are ion exchange, reverse osmosis, adsorption, and electrodialysis. However, the high costs of these technologies limit their applications (Shrimali et al., 2001; Zhou et al., 2016). Biological denitrification process is a well-developed approach and considered cost-effective to remove nitrate from wastewaters, due to a high efficiency and no production of by-products (Foglar et al., 2005; Zhang et al., 2015; Zhou et al., 2016). Biological denitrification can be performed heterotrophically or autotrophically. Heterotrophic denitrifiers require carbon source, making this process not suitable for the treatment of low C/N ratio water. Addition of external carbon source would result in extra operating costs as well as the presence of organic residues that generate secondary organic pollution (Zhang et al., 2015).

Fe(II)-mediated autotrophic denitrification is an alternative biotechnology to simultaneously remove nitrate and recover iron through the formation of Fe(III) precipitates (Straub et al., 1996) and can be particularly efficient for the treatment of low-organic wastewaters (Kiskira et al., 2017a, b), even in the presence of metals (Papirio et al., 2014; Zou et al., 2014, 2015). At neutral pH, autotrophic denitrification yields energy, since the redox potential of ferrous/ferric couples is about +200 mV and is lower than that of nitrate/nitrogen gas (+710 mV) (Hedrich et al., 2011).

The first microorganisms capable of maintaining biological nitrate-dependent Fe(II) oxidation were discovered 20 years ago (Straub et al., 1996). Since then all the other microbial species reported to maintain Fe(II)-driven denitrification have been reviewed in detail by Kiskira et al., (2017a). However, limited work has been done in the field of wastewater treatment, especially within continuous-flow bioreactor applications. An up-flow anaerobic sludge bed reactor (UASB) of 0.8 L, inoculated with anaerobic granular sludge, was used by Zhang et al. (2015). The process was observed at its steady state during 32-47 d

with nitrate and Fe(II) removal reaching 45 and 44%, respectively. The efficiency of the process decreased during the experimentation due to the decrease of hydraulic retention time (HRT) and pH. In another study, two 420 mL continuous-flow sand packed bed columns, inoculated with activated sludge and operated with an HRT of 24 h were used for Fe(II) and As(III) oxidation coupled to autotrophic denitrification (Sun et al., 2009). However, acetate was used as an additional electron donor and the process was mainly addressed to the immobilization of As onto the biogenic ferric (Fe(III)) (hydr)oxides produced. Zhou et al. (2016) used a continuous-upflow biofilter packed with sponge iron and inoculated with *Microbacterium* sp. W5, a nitrate reducing and Fe(II) oxidizing strain. The maximum nitrogen removal efficiency was about 90% with an HRT of 24 h.

In this work, Fe(II) mediated autotrophic denitrification was investigated as a biotechnology for nitrate removal using two identical up-flow packed bed reactors (PBRs) made of Plexiglas and fed with different influent nitrate concentrations. The bioreactors were seeded with the *Thiobacillus* mixed culture, in order to optimize the operating parameters such as the hydraulic retention time (HRT) and the nitrate loading rate and evaluate their influence on nitrate removal and Fe(II) oxidation.

6.2 Materials and methods

6.2.1 Reactor design

Two identical up-flow PBRs were used for the continuous flow experiments. Figure 6.1 shows the physical schematic of the reactors. The reactors were made up of Plexiglas, with height and internal diameter being 24 and 8 cm, respectively, corresponding to an effective volume of 0.98 L and a total volume of 1.50 L. The filler zone, which had a height of 16 cm, consisted of granular activated carbon, as reported by Papirio et al. (2014), which was used as biomass carrier. Glass pearls with a diameter in the range 0.4-1.0 cm were placed in the bottom of the reactors to avoid blocking of the inlet with activated carbon. The effluent was discharged by gravity from an outlet port located 3.5 cm above the top of filler. The reactors were operated in continuous mode and samples were collected from the outlet port.

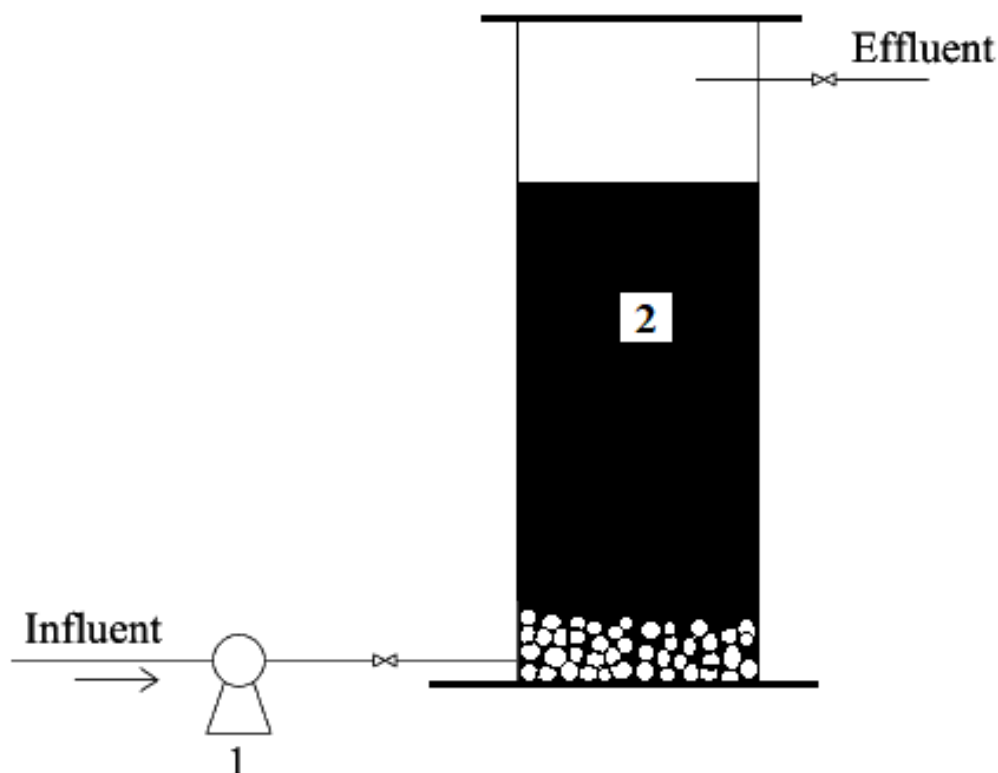


Figure 6.1: Schematic of the continuous up-flow packed bed reactors used for Fe(II)-driven denitrification: 1 peristaltic pump; 2 filler

6.2.2 Inoculum and reactor start-up

The *Thiobacillus*-dominated mixed culture, enriched on both thiosulfate and iron as electron donors (chapters 3 and 4), was inoculated into the reactors. The PBRs were seeded with 50 mL of the microbial culture, having an initial VSS concentration of 200 mg VSS/L. After seeding, the bioreactors were flushed with nitrogen. The operation of the reactors started with an “acclimation phase” performed under batch conditions for 1 week. The feed Fe(II) and NO_3^- concentrations were 600 and 120 mg/L, respectively. The culture medium characteristics were described in section 3.2.1.

6.2.3 Feed synthetic wastewater and PBRs operation

A synthetic wastewater was continuously fed from the bottom of the reactors by a peristaltic pump. Initially, the two PBRs (Reactor 1 and 2) were fed with 120 and 60 mg/L of nitrate and 600 and 300 mg Fe(II)/L, respectively, in order to maintain a Fe(II): NO_3^- molar ratio of 5:1. EDTA was supplemented at a EDTA:Fe(II) molar ratio of 0.5:1. The pH and

temperature were 6.5-7.7 and 22±2°C, respectively. The feed solution contained the basal medium and trace elements, as described in section 3.2.1, and were flushed with N₂ prior to being fed to the PBRs.

The operation was divided into 5 periods (Table 6.1) along 153 days.

Table 6.1: PBRs operation periods and conditions

	Time (d)	Influent NO ₃ ⁻ concentration (mg/L)		HRT (h)	Comments
		PBR1	PBR2		
Period I	0-63	120	60	31	
	64-76	250	180	31	
Period II	76	250	180	31	Addition of 50 ml biomass in PBR1
	76-92	250	180	31	
Period II, III	90-98	250		31	PBR2 operation was stopped due to a leakage
	93-115	250	180	28	
Period III	108		250	28	
Period IV	116-139	250	215	24	
Period V	140-153	250	180	20	

The feed nitrate concentration was increased to 250 and 180 mg/L for reactor 1 (PBR1) and reactor 2 (PBR2), respectively, on day 64. However, PBR2 operation was stopped after 90 d due to a leakage, and continued after 1 week with a feed NO₃⁻ concentration of 250 mg/L. HRT was gradually decreased from 31 to 20 h for both PBRs from day 93 to day 153. On day 116, the operation of the two PBRs was differentiated. In PBR1, the NO₃⁻ concentration was maintained stable at 250 mg/L, in order to evaluate the effect of higher nitrate loading rates at decreasing HRTs. Conversely, in PBR2 the nitrate loading rate remained stable, by gradually decreasing the influent nitrate concentration from 250 to 180 mg/L according to the HRT used. On day 76, 50 mL of biomass was inoculated for enhancing the nitrate removal in PBR1. The process was monitored by sampling both the liquid phase and biomass.

6.2.4 Analytical methods

Ferrous iron, nitrate and pH were analyzed every 48 h. The NO_3^- concentration was analyzed by ion chromatography (IC) with chemically suppressed conductivity using a 883 Basic IC Plus system equipped with a Metrosep A Supp 5-150/4.0 column and a 863 Compact IC Autosampler (Metrohm, Switzerland). The liquid samples were filtered with 0.22 μm syringe cellulose membranes (EMD Millipore, USA) prior to IC analysis. Ferrous iron was quantified photometrically by using UV-1800 240V IVDD UV spectrophotometer (Shimadzu, Japan), following the analytical method with 1,10 phenanthroline reported by Ahoranta *et al.*, (2016). Fe(II) determination was performed immediately after the sampling, for avoiding Fe(II) chemical oxidation. VSS were analyzed according to the standard methods (APHA, 1992).

6.3 Results

Two identical PBRs were operated in order to investigate the performance of Fe(II)-mediated autotrophic denitrification under continuous-flow conditions. Nitrate removal and Fe(II) oxidation were evaluated using the *Thiobacillus*-mixed culture in PBR1 and PBR2 operated for 153 days under different operational conditions (Fig. 6.2a, Fig. 6.3a). Figure 6.2b,c and Figure 6.3b,c show nitrate removal and Fe(II) oxidation in PBR1 and PBR2, respectively.

Table 6.2 reports the nitrate removal efficiency and rates as well as Fe(II) oxidation efficiency and rates at the end of each experimental period in both PBRs. Throughout the operation of reactors, no nitrite was detected as intermediate of denitrification in agreement with other studies (Straub *et al.*, 1996; Weber *et al.*, 2006; Zhang *et al.*, 2015).

As depicted in Fig. 6.2b and Fig 6.3b, during period 1 (0-63 days) nitrate removal and Fe(II) oxidation were 70 and 85%, respectively, with an effluent NO_3^- concentration that gradually decreased to 37 mg NO_3^-/L in PRB1. Fe(II) oxidation occurred with a Fe(II): NO_3^- molar ratio in the range 4.7 to 7.4, indicating that part of Fe(II) oxidation was not associated with nitrate removal as Fe(II) was most likely oxidized due to the chemical reaction with residual DO.

During 0-63 d, Fe(II)-mediated denitrification resulted in an effluent nitrate concentration decreasing till to 36 mg NO_3^-/L , corresponding to 41% of nitrate removal in

PBR2. Thus, the higher feed nitrate concentration used for the PBR1 operation led to a higher nitrate removal by the *Thiobacillus*-mixed culture. Fe(II) oxidation and nitrate removal reached up to 70 and 85% with a specific nitrate removal rate of 211.7 mg/(g VSS·d).

During the operation of the PBRs in Period II (64-92 d), the influent nitrate concentration was doubled to 240 mg NO₃⁻/L in PBR1, and increased by 3 times up to 180 mg NO₃⁻/L in PBR2. As a result, nitrate removal decreased from 70 to 62% in PBR1 only one day after the variation of the operating conditions. On the contrary, Fe(II)-driven denitrification was enhanced in PBR2. Nitrate removal increased by 20% at 64 d corresponding to a nitrate removal and Fe(II) oxidation of 64% and 72%, respectively. Before the occurrence of a leakage at the end of Period II (day 92), nitrate removal and Fe(II) oxidation further increased to 80 and 94%, respectively, in PBR2.

In order to stimulate denitrification in PBR1, 50 mL of biomass was inoculated on day 76. The effluent nitrate concentration gradually decreased and nitrate removal reached 88% on day 92. Fe(II) oxidation was enhanced up to 93%. The specific nitrate removal rate was as high as 317.5 mg/(g VSS·d) after 92 d (end of period II).

Starting from period III (93-115 d), HRT was gradually decreased in both PBRs with the aim to evaluate the effect of HRT on the denitrification process efficiency. At an HRT of 28 h, nitrate removal decreased by only 3% in PBR1. From that point, until the end of the period III, the nitrate removal slightly increased and reached 88%, as at the end of the Period II, indicating that the denitrifiers adapted to the new operating conditions. The specific nitrate removal rate was 341.0 mg/(g VSS·d). During periods IV (116-139 days) and V (140-153 days), Fe(II)-mediated autotrophic denitrification was stable with a nitrate removal and a Fe(II) oxidation being at 86±2% and 96±3%, respectively, till day 153. Denitrification was not affected when decreasing the HRT from 28 to 24h during Period IV (days) and from 24 to 20 h during Period V (140 to 153 d). The specific nitrate removal rate reached up to 337.1 mg/(g VSS·d) at the end of the experiment.

At the beginning of Period III (day 93), the influent nitrate concentration was increased from 180 to 250 mg/L for enhancing the process after the leakage occurred in PBR2. Nitrate removal and Fe(II) oxidation, however, constantly decreased and were 10 and 34% lower on day 115 than those observed on day 92.

Table 6.2: Nitrate removal (%), specific nitrate removal rates, Fe(II) oxidation efficiency and rates obtained in PBR1 and PBR2 operated for 153 days under different operating conditions

	Time (d)	Nitrate Removal (%)		Fe(II) oxidation (%)		Specific nitrate removal rate mg/(g VSS·d)		Specific Fe(II) oxidation rate mg/(g VSS·d)	
		PBR1	PBR2	PBR1	PBR2	PBR1	PBR2	PBR1	PBR2
Period I	0-63	70	41	85	69	211.7	81.3	1246.6	661.5
Period II	64-92	88	80	93	94	317.5	315.6	1634.6	1708.1
Period III	93-115	87	70	94	74	341.0	310.7	1606.2	1599.4
Period IV	116-139	87	69	95	72	344.0	293.0	1417.1	1395.5
Period V	140-153	85	67	95	69	337.1	272.4	1174.0	1361.2

In Period IV and V, the HRT and the feed nitrate concentration were simultaneously decreased in order to maintain a stable nitrate loading rate in PBR2. Denitrification was observed at its steady state during days 116-153 with a nitrate removal and Fe(II) oxidation of $66\pm4\%$ and $70\pm2\%$, respectively. A lower feed NO_3^- concentration did not enhance the nitrate removal efficiency. The specific nitrate removal rate decreased to 272.4 mg/(g VSS·d).

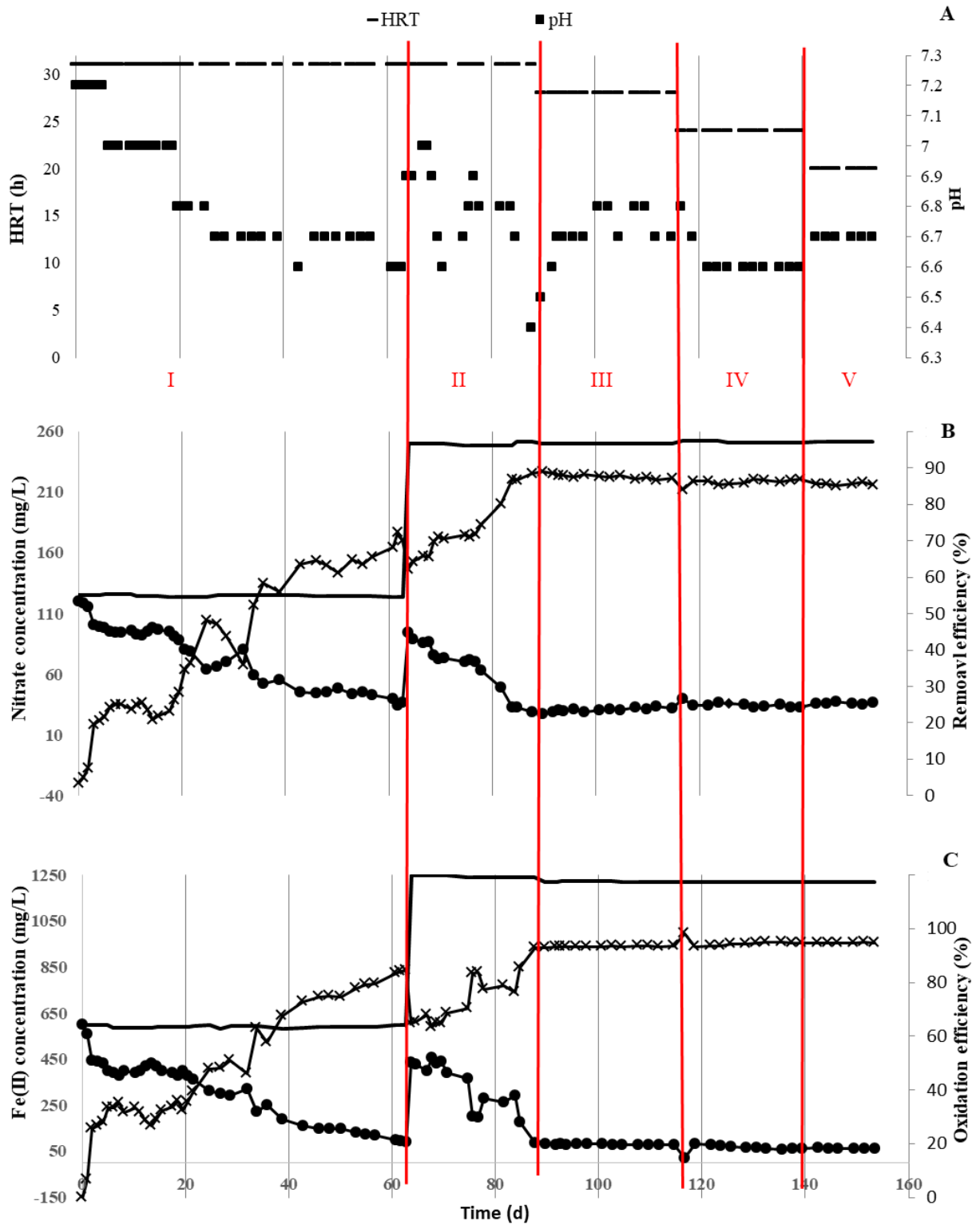


Figure 6.2: A) pH and HRT, B) Influent nitrate concentration (—), effluent nitrate profile (●) and nitrate removal efficiency (x), C) Influent Fe(II) concentration (—), effluent Fe(II) profile (●) and Fe(II) oxidation efficiency (x) in PBR1.

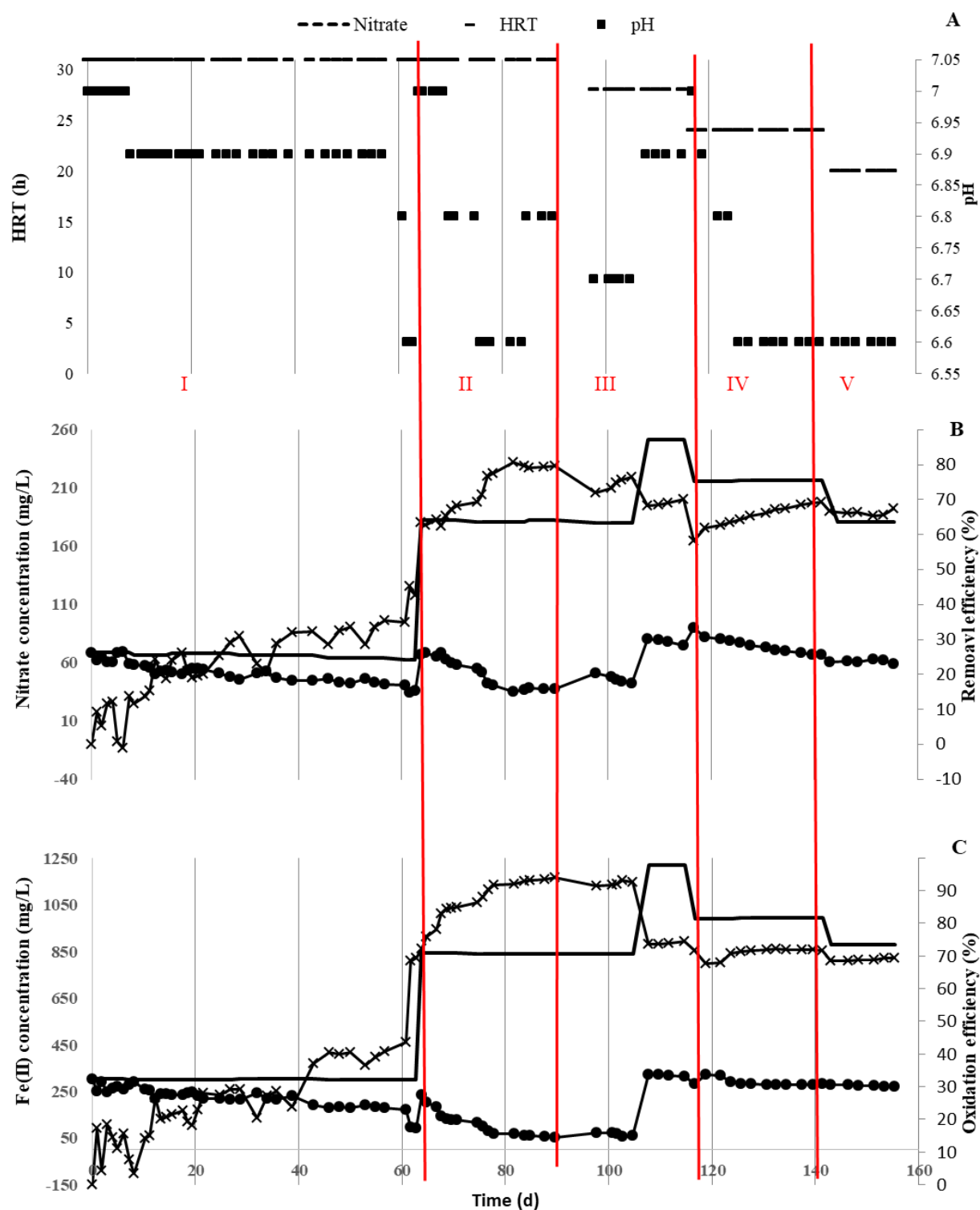


Figure 6.3: A) pH and HRT, B) Influent nitrate concentration (—), effluent nitrate profile (●) and nitrate removal efficiency (x), C) Influent Fe(II) concentration (—), effluent Fe(II) profile (●) and Fe(II) oxidation efficiency (x) in PBR2.

6.4 Discussion

6.4.1 Nitrate removal and Fe(II) oxidation in continuous-flow packed-bed reactors

In this study, the highest nitrate removal rates and efficiency were observed at the highest feed nitrate concentration. This is in contrast with what reported by the only two studies on the continuous-flow application of Fe(II)-mediated autotrophic denitrification (Zhang et al., 2015; Zhou et al., 2016). Zhang et al. (2015) reported that feed concentrations higher than 130 mg NO_3^-/L resulted in a lower nitrate removal in an up-flow anaerobic sludge bed reactor (UASB) with a total working volume of 0.8 L, inoculated with granular sludge collected from a full-scale UASB reactor treating a wastewater from a paper mill. An increase of the influent nitrate concentration from 30 to 170 mg NO_3^-/L led to an almost 70% lower nitrate removal efficiency. Similarly, in a continuous flow up-flow biofilter packed with sponge iron and inoculated with a pure culture of *Microbacterium* sp. W5, nitrate removal decreased by approximately 10% when the influent nitrate concentration was increased from 30 to 100 mg NO_3^-/L (Zhou et al., 2016).

The reason for this discrepancy between the current study and those present in the scientific literature might be that the biofilm used in this study was longer enriched on Fe(II) and was capable of reaching a higher nitrate removal at higher influent nitrate concentrations. At lower feed concentrations, the process was less stimulated and the nitrate removal efficiency remained stable. The *Thiobacillus*-mixed culture had previously been enriched on Fe(II) in batch experiments (chapters 3 and 4), and then was inoculated to the PBRs for 7 days using a feed nitrate concentration of 120 mg NO_3^-/L , thus 4-fold higher than that used by Zhang et al. (2015) and Zhou et al. (2016). Despite an enrichment on NO_3^- and Fe(II) was performed by Zhou et al. (2016), an acclimation of biomass in the reactors was not performed, thus leading to a lower nitrate removal performance.

Another possible reason of the better Fe(II)-driven denitrification performance at the highest influent nitrate concentrations achieved in this study was likely due to the presence of EDTA in an EDTA:Fe(II) molar ratio of 0.5:1. EDTA chelates Fe(II) and minimizes the chemical oxidation of Fe(II) with residual oxygen (Chakraborty and Picardal, 2013). Moreover, EDTA prevents the cell encrustation by the Fe(III) hydroxide mineral formation, allowing the activity of microbes (Shelobolina et al., 2003; Chakraborty et al., 2011). Zhou et al. (2016) initially operated a packed bio-filter with a feed Fe(II) concentration up to 1500-1800 mg Fe(II)/L, without supplementing EDTA. The authors reported that Fe(II)

concentrations higher than 800 mg Fe(II)/L resulted in a decrease of nitrate removal efficiency. Subsequently, when EDTA was added at 10 g/L, nitrate removal increased back to 90% even at a feed Fe(II) concentration of 1100 Fe(II)/L.

During the PBR operation, the average molar ratio between the consumed Fe(II) and nitrate was 5.1 and 4.8 in PBR1 and PBR2, respectively, which is in agreement with theoretical amount suggested by Sorensen (1987) (see eq. 2.1). Zhang et al. (2015) reported a lower molar ratio, i.e. 2.9. Similarly, a Fe(II):NO₃⁻ molar ratio, in the range 2.5-5.0, has been reported in other studies with activated sludge (Nielsen and Nielsen, 1998; Li et al., 2014). A lower Fe(II)/NO₃⁻ ratio than 4.0 indicates the production of intermediates such as nitrite and nitrous oxide, while a molar ratio higher than 7.5 results in production of ammonium (Li et al., 2014).

6.4.2 Comparison with the previous batch experiments and other denitrification processes

The implementation of Fe(II)-mediated autotrophic denitrification in continuous-flow experiments resulted in higher specific nitrate removal rates compared to those obtained in the previous batch experiments (Section 3.3), due to a longer acclimation of the biomass and a better denitrifying performance of the biofilm in continuous-flow experiments, as also reported by Zhang et al. (2015). A 32 and 26 times higher specific nitrate removal rate, i.e. 337.1 and 272.4 mg/(g VSS·d) was achieved for PBR1 and PBR2, respectively, than those observed in the same above mentioned batch tests.

During the steady state the maximum volumetric loading rates (VLRs) were 280 mg NO₃⁻/(L·d) and 210 mg NO₃⁻/(L·d) for PBR1 and PBR2, respectively. The maximum volumetric removal rates (VRR) of nitrate were 240 mg NO₃⁻/(L·d) for PBR1 and 160 mg NO₃⁻/(L·d) for PBR2 and were recorded at steady state. Zhang et al., (2015) reported a similar maximum VRR of 190 mg NO₃⁻/(L·d), but the VRR at steady state was as low as 73 mg NO₃⁻/(L·d), because of the pH decrease. A much lower VRR, i.e. 25 mg NO₃⁻/(L·d), was observed in Zhou et al., (2016).

Compared to other denitrification processes, Fe(II)-driven autotrophic denitrification showed to be a promising process, but with still lower efficiencies than other well-established technologies. For instance, sulfur-driven autotrophic denitrification VRRs in the range 50-560 mg NO₃⁻/(L·d) have been observed in continuous flow experiments (Park and Yoo, 2009; Show et al., 2013), i.e. higher by approximately 55% than the highest obtained in this study. Heterotrophic denitrification with ethanol in denitrifying cultures resulted in 400 mg NO₃⁻/(L·d) of VRR in a similar bioreactor configuration (Papirio et al., 2014). However, the results of this study are quite encouraging and stimulate further studies for the optimization of Fe(II)-mediated autotrophic denitrification as a future biotechnology for nitrate removal.

6.4.3 Effect of pH and HRT

The pH in the influent was maintained at 7.7 during the entire operation of both PBRs, as Fe(II)-mediated autotrophic denitrification produces acidity and a feed pH higher than 7.0 is recommended (Straub et al., 1996; Weber et al., 2006; Zhang et al., 2015). During denitrification, the effluent pH gradually decreased to 6.4 without affecting the process. Zhang et al. (2015) reported that a pH lower than 3.0 in the reactor resulted in a decrease of nitrate removal efficiency by 80%. However, nitrate removal was higher than 95% as long as the effluent pH was higher than 6.0. In acidic environments, Fe(II) is not favored to act as electron donor to yield energy as under circumneutral conditions, since the redox potential of ferrous/ferric couple in acidic liquors is approximately +770 mV, i.e. much higher than +430 mV of the NO₃⁻/NO₂⁻ couple (Hedrich et al., 2011).

In this study, HRT was decreased from 31 h to 20 h. Denitrification was not affected when decreasing the HRT, indicating that further continuous-flow experiments should be performed in order to reduce the HRT. In contrast, the efficiency of the process was negatively affected due to the decrease of HRT in Zhang et al. (2015). At HRT 18 h, nitrate removal and Fe(II) oxidation were above 95%, however when HRT was only slightly reduced to 17 h, 60 and 38% of nitrate removal and Fe(II) oxidation, respectively, were observed. A stable HRT of 24 h was used in Zhou et al. (2016), as the effect of decreasing HRT was not investigated.

6.5 Conclusions

Fe(II) mediated autotrophic denitrification in continuous flow experiments was maintained in two PBRs with a nitrate removal efficiency higher than 65% also at an HRT of 20 h. During the steady state the maximum VLR and VRR were 280 and 240 mg NO₃⁻/(L·d) for PBR1 and 210 and 160 mg NO₃⁻/(L·d) for PBR2. Results showed that the nitrate removal rate increased at increasing feed nitrate concentrations. At the steady state, nitrate removal and Fe(II) oxidation were 86±2% and 95.5±2.5%, respectively, in PBR1, whereas 65.5±3.5% and 70.0±2%, in PBR2. The lower initial feed NO₃⁻ concentration resulted in lower efficiencies in PBR2.

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CHAPTER 7

General discussion and future perspectives

7.1 Introduction and objectives

Water quality is vital for humans and ecosystems. The drinking water standard set by the World Health Organization (WHO) (1993) for nitrate is 50 mg/L. Nitrate is the most common pollutant in water resources, due to the extensive use of fertilizers in agriculture and the discharges of raw and treated wastewater (Shrimali et al., 2001). Therefore, the water should be treated properly and wastewater treatment be done carefully. The conventional methods for nitrate removal of wastewaters are ion exchange, adsorption, chemical treatment, membrane technology, and biological treatment (Park and Yoo, 2009). However, the physicochemical methods are expensive and the biological treatment application has mainly been focused on heterotrophic denitrification, which is not desirable for the treatment of low-organic wastewaters (Shrimali et al., 2001; Park and Yoo, 2009). Furthermore, in mining wastewaters, nitrate co-occurs with valuable metals, including iron that can be recovered by using biotechnologies, such as autotrophic denitrification (Papirio et al., 2013; Papirio et al., 2014).

Fe(II) iron-mediated autotrophic denitrification is a new bioprocess for nitrate removal and removal/recovery of iron from wastewaters (Kiskira et al., 2017a). The use of denitrifying Fe(II) oxidizers results in the reduction of nitrate to nitrogen gas and the bioprecipitation/biorecovery of Fe(III) (hydr)oxides that can be subsequently removed and recovered, with the possible co-precipitation or adsorption of other metals (Hohmann et al., 2009; Ahoranta et al., 2016). Recently, the process has gained scientific interest over classical heterotrophic denitrification especially for the treatment of industrial wastewaters containing very low amount of biodegradable COD (Zhang et al., 2015). Despite several detailed studies on the microbiology of these microorganisms capable for nitrate-dependent ferrous oxidation, little research has been done in order to investigate the Fe(II) mediated autotrophic denitrification as a biotechnology for nitrate removal and iron recovery.

This research investigated the efficiency of Fe(II)-mediated autotrophic denitrification in terms of Fe(II) oxidation and nitrate removal with different microbial cultures. An extensive review of the current knowledge about Fe(II)-mediated autotrophic denitrification was conducted in Chapter 2, presenting a short summary of iron biogeochemistry and the environmental concerns of Fe(II) and nitrate, the characteristics of the Fe(II)-oxidizing denitrifiers, the operating conditions adopted in the existing studies, the characteristics of the produced Fe(III) hydr(oxides) and the influence of other metals on the process. Finally, the use of pyrite as an alternative electron donor is discussed. Obviously, there are several factors

affecting the process: the microbial cultures used, pH, the EDTA/Fe(II) ratio, the presence of other heavy metals, the operation of the experiments (batch or continuous-flow mode) and how long and on which electron donors the microorganisms were enriched. All these factors were taken into account in this PhD research for designing suitable bioreactors for Fe(II)-mediated autotrophic denitrification. The major findings from individual chapters of this PhD are shown in Figure 7.1.

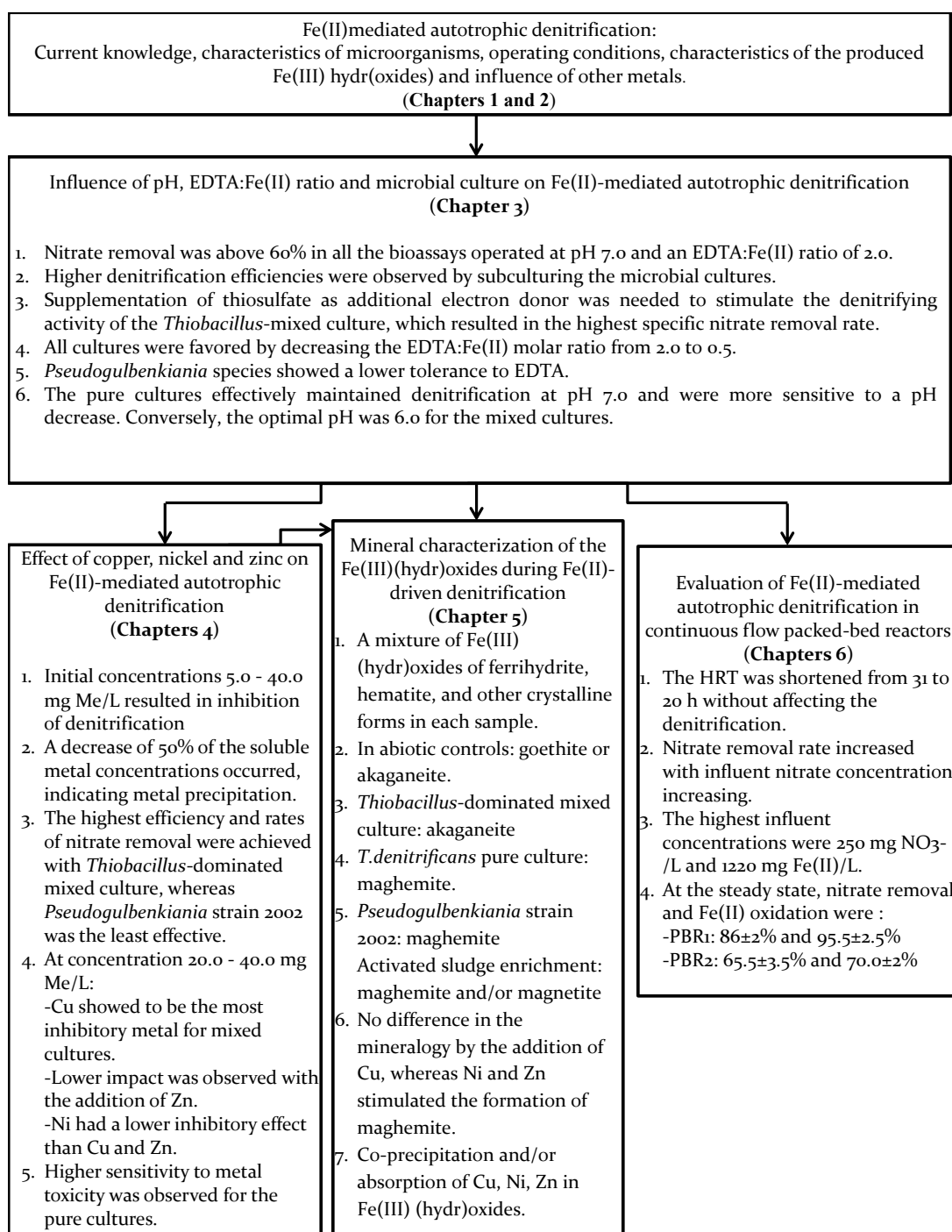


Figure 7.1: Summary of the major findings of this PhD research

7.2 Major research findings

7.2.1 Influence of pH, EDTA:Fe(II) ratio and microbial culture on Fe(II)-mediated autotrophic denitrification

The objectives of this work was to investigate the efficiency of Fe(II)-mediated autotrophic denitrification in terms of Fe(II) oxidation and nitrate removal with different microbial cultures in batch bioassays and evaluate the effects of decreasing pH and EDTA/Fe(II) ratios on the process. Fe(II)-mediated autotrophic denitrification was investigated in batch experiments with different microbial cultures (Kiskira et al, 2017b). Table 3.2 reports the specific nitrate and Fe(II) oxidation rates with all microbial cultures. Nitrate removal was above 60% in all the bioassays operated at pH 7.0 and an EDTA:Fe(II) ratio of 2.0. In all experiments, no nitrite was detected as intermediate of denitrification, indicating a complete nitrate reduction to N₂. Initially, experiments were conducted with the use of Fe(II) as sole electron donor (Fig. 3.1a). The pure *T. denitrificans* culture, activated sludge inoculum and *Pseudogulbenkiania* strain 2002 were capable of maintaining Fe(II)-mediated autotrophic denitrification. Conversely, denitrification did not occur in the bioassays with the *Thiobacillus*-mixed culture.

Then, the capability for Fe(II)-mediated autotrophic denitrification of the previously Fe(II)-enriched *Thiobacillus*-mixed culture and *T. denitrificans* culture was investigated by also supplementing thiosulfate as additional electron donor (Fig. 3.2). Both the *Thiobacillus*-mixed and pure *T. denitrificans* cultures were stimulated in the presence of thiosulfate. After enriching and acclimating the *Thiobacillus*-mixed and pure *T. denitrificans* cultures on thiosulfate, Fe(II)-based denitrification was again investigated with Fe(II) as sole electron donor. The use of a more enriched *Thiobacillus*-mixed culture resulted in a higher biological Fe(II) oxidation coupled to denitrification (Fig. 3.1b). The specific nitrate removal rate significantly increased and reached 8.6 mg/(g VSS·d). Fe(II) oxidation and NO₃⁻ reduction were 68 and 83%, respectively.

The rates of Fe(II)-mediated autotrophic denitrification obtained in this work were lower than those reported in similar batch experiments aimed at investigating classical heterotrophic denitrification or sulfur-driven autotrophic denitrification. Di Capua et al. (2016), reported a N-NO₃⁻ removal rate of 52.2 mg·L⁻¹·d⁻¹, i.e. 3-fold higher than the highest achieved in this study by sulfur-driven autotrophic denitrification, whereas Papirio *et al.* (2014) observed a nitrate removal rate up to approximately 400 mg·L⁻¹·d⁻¹ by using denitrifying cultures enriched on ethanol. However, the implementation of Fe(II)-mediated

autotrophic denitrification in continuous-flow experiments resulted in higher nitrate removal rates, due to a longer acclimation of the biomass and a better denitrifying performance of the biofilm in continuous-flow experiments, as previously reported by Zhang et al. (2015). In this study, a 32 and 26 times higher specific nitrate removal rate, i.e. 337.1 and 272.4 mg/(g VSS·d) was reported for PBR1 and PBR2, respectively. In comparison with heterotrophic and sulfur-based autotrophic denitrification, Fe(II)-mediated autotrophic denitrification does not result in nitrite and this represents a major advantage as nitrite is reported to be inhibitory for many denitrifiers and nitrogen is thus entirely removed from the liquid phase (Straub et al., 1996; Zhang et al., 2015).

EDTA is used to improve Fe(II) solubility and bioavailability and has been used as chelating agent in many other studies (Kumaraswamy et al., 2006; Chakraborty et al., 2011; Chakraborty and Picardal, 2013; Kanaparthi et al., 2013; Kanaparthi and Conrad, 2015; Klueglein et al., 2015). Fe-EDTA optimizes the process as the abiotic reactions are minimized (Chakraborty and Picardal, 2013) and prevents Fe(III) hydroxide mineral formation allowing the activity of microbes (Zhou, et al., 2016). The amount of EDTA is of major importance in Fe(II)-based denitrification. Microbial activity can be influenced by the molar EDTA:Fe(II) ratio as microbial cultures differently tolerate the inhibitory effects of free EDTA and Fe-EDTA species (Klueglein et al., 2015). Free EDTA is generally the most toxic EDTA form to bacteria as it disrupts the cell membranes (Oviedo and Rodríguez, 2003). The NO₃⁻ removal efficiency achieved at EDTA:Fe(II) ratios of 2.0, 1.0 and 0.5 was as shown in Fig. 3.3. The most significant effect of the EDTA concentration was observed with the *Pseudogulbenkiania* strain 2002 culture, indicating the lower tolerance of this species to EDTA. The lowest molar EDTA:Fe (II) ratio resulted in an almost double nitrate removal rate compared to that achieved with an EDTA:Fe(II) ratio of 2.0. This study demonstrated that all the investigated cultures were alleviated by decreasing the EDTA concentration.

pH also is a very important parameter affecting both microbial activity and iron speciation/solubility (Hedrich et al., 2011). At pH < 4.0, Fe(II) is more stable but the inhibition of most denitrifiers occurs (Johnson et al., 2012). The known Fe(II)-oxidizing denitrifiers are neutrophilic (Hedrich et al., 2011), and therefore up to now the majority of studies was performed at pH between 6.0 and 8.0 (Kiskira et al., 2017a) (Chapter 2). During this research, the effect of decreasing pH from 7.0 to 5.0 was assessed. The activity of the *Thiobacillus*-mixed and activated sludge enrichment was enhanced by decreasing pH from 7.0 to 6.0 (Fig. 3.3), whereas the two pure cultures of *T. denitrificans* and *Pseudogulbenkiania* strain 2002 demonstrated to be less tolerant to the decreasing pH. Both

chemical and biological Fe(II) oxidation coupled to denitrification were repressed at pH 5, resulting in lower Fe(II) oxidation rates.

7.2.2 Effect of copper, nickel and zinc on Fe(II)-mediated autotrophic denitrification

The effect of Cu, Ni, Zn on Fe(II)-mediated autotrophic denitrification in the efficiency and rates of nitrate removal and how the different microbial cultures tolerated the effect of metals were investigated in another set of batch bioassays. The microbial activity of denitrifying microorganisms can be either stimulated or inhibited by the presence of heavy metals. Metals can enhance metabolic degradation, whereas at higher concentrations can change the microbial enzyme conformation and block essential functional groups (Gikas, 2007; Giller et al., 2009).

During this study, a decrease of the soluble Cu, Ni and Zn concentration occurred in the first 4 d in all the experiments (Fig. 4.1), likely due to metal precipitation and biosorption (Crane et al., 2010). Figure 4.2 shows the nitrate removal efficiency (%) achieved after 10 d in the bioassays seeded with the four microbial cultures at different initial metal concentrations. All metals negatively affected denitrification, whereas no stimulation was observed even at the lowest Ni, Cu and Zn concentrations. The highest efficiency and rates of nitrate removal were achieved with *Thiobacillus*-dominated mixed culture, whereas *Pseudogulbenkiania* strain 2002 was the least effective. At an initial concentration of 20.0 - 40.0 mg Me/L, Cu showed to be the most inhibitory metal for mixed cultures, with an inhibition of nitrate removal in the range 33-66%. A lower impact was observed with the addition of Zn that resulted in 17 to 41% inhibition. Ni had the lowest inhibitory effect than Cu and Zn. A 5-34% inhibition at concentrations of 20.0 to 40.0 mg Ni/L was recorded. A higher sensitivity to metal toxicity was observed for the pure cultures. The results obtained in this study were similar compared to the existing literature for denitrifying bacteria (Ochoa-Herrera et al. 2011; Zou et al. 2013). However, this was the first study reporting the effect of these metals on Fe(II) mediated autotrophic denitrification.

7.2.3 Mineral characterization of the Fe(III)(hydr)oxides during Fe(II)-driven denitrification

The recovery of iron and other heavy metals by the formation of Fe(III) (hydr)oxides is an important application of some microbial processes such as Fe(II)-mediated autotrophic

denitrification (Hohmann et al., 2009; Sun et al., 2009; Ahoranta et al., 2016; Xiu et al., 2016). Mineral characterization of the precipitates formed during the Fe(II) mediated autotrophic denitrification was performed by X-ray fluorescence (XRF), Raman spectroscopy, scanning electron microscopy equipped with energy dispersive X-Ray Analyser (SEM-EDX) and Fourier transformation infrared spectroscopy (FTIR). The evaluation of the particular Fe(III) precipitates is important in order to decide on the “reuse strategy” to adopt and the applications in which to use them. Some of the major applications are pigments for paints and the construction industry, magnetic pigments and ferrites, catalysts for industrial syntheses, adsorbents for water and gas purification and for low level radioactive waste decontamination, production of photochemicals and fertilizers (Cornell and Schwertmann, 2003).

FTIR and Raman spectroscopy of the samples formed during Fe(II) mediated autotrophic denitrification showed a mixture of Fe(III) (hydr)oxides in each sample. The observed bands were similar, indicating that the medium composition and the incubation conditions are important factors for Fe(III) (hydr)oxides formation. However, differences between the abiotic controls and the precipitates formed from different microorganisms were observed, showing that different microorganisms resulted in different biogenic Fe(III) (oxyhydr)oxides minerals, in agreement with other studies (Senko et al., 2005; Posth et al., 2014). Ferrihydrite was most probably present in all the biogenic precipitates, as also reported by many authors (Straub et al., 1996, 2004; Lack et al., 2002; Senko et al., 2005; Hohmann et al., 2009; Chakraborty et al., 2011, Zhao et al., 2013)

In abiotic controls, the chemical Fe(II) oxidation resulted in hematite formation and goethite or akaganeite. Precipitates of the experiments carried out with the *Thiobacillus*-dominated mixed culture were a mixture of hematite, akaganeite and/or ferrihydrite. When *T. denitrificans* was used, on top of hematite and/or ferrihydrite, maghemite was observed. The use of pure culture of *Pseudogulbenkiania* strain 2002 resulted in hematite and maghemite formation and the activated sludge enrichment allowed the production of hematite and maghemite and/or magnetite (and/or ferrihydrite). In agreement, the formation of a mixture of Fe(III) oxides dominated by crystalline hematite by Fe(II) mediated autotrophic denitrification has also been reported by Sun et al. (2009). The use of Cu, Ni and Zn resulted in very similar Fe(III) (hydr)oxides than those obtained in the absence of metals. However, the addition of Ni and Zn stimulated the formation of maghemite. No other study investigated the effect of the addition of Cu, Ni and Zn on the formation of biogenic minerals by nitrate-

dependent Fe(II)-oxidizing microorganisms. Precipitates obtained contained Cu, Ni and Zn, indicating absorption and/or co-precipitation with Fe(III)(hydr)oxides.

7.2.4 Evaluation of Fe(II)-mediated autotrophic denitrification in continuous flow packed-bed reactors

In this study, the highest nitrate removal rates and efficiency were observed at the highest feed nitrate concentration, in contrast with what was reported by the only two studies on the continuous-flow application of Fe(II)-mediated autotrophic denitrification (Zhang et al., 2015; Zhou et al., 2016). The biofilm used in this study was longer enriched on Fe(II) and was capable of reaching a higher nitrate removal at higher influent nitrate concentrations. Furthermore, the addition of EDTA likely prevented cell encrustation by the Fe(III) hydroxide mineral formation, allowing the activity of microbes (Shelobolina et al., 2003; Chakraborty et al., 2011).

At the steady state, nitrate removal and Fe(II) oxidation were $86.0 \pm 2.0\%$ and $95.5 \pm 2.5\%$, respectively, in PBR1, whereas $65.5 \pm 3.5\%$ and $70.0 \pm 2.0\%$, in PBR2. The lower initial feed nitrate concentration resulted in a lower efficiency in PBR2. The effluent pH decrease did not affect the performance of the process. The lowest HRT used was 20 h, as other studies on continuous flow experiments that reported a minimum HRT in the range 18-24 h (Zhang et al., 2015; Zhou et al., 2016). However, the operation of the two PBRs needs further investigation as the process has the potential to tolerate even lower HRT values.

Finally, if compared to other denitrification processes, Fe(II) driven autotrophic denitrification showed quite high efficiencies. For instance, nitrate removal rates during sulfur-driven autotrophic denitrification have been reported to be in the range 50-560 mg $\text{NO}_3^-/(\text{L} \cdot \text{d})$ and heterotrophic denitrification with ethanol resulted in nitrate removal rates of approximately 400 mg $\text{NO}_3^-/(\text{L} \cdot \text{d})$ (Park and Yoo, 2009; Show et al., 2013 Papirio et al., 2014).

7.3 Future research perspectives

Based on the results obtained during the various batch and continuous flow experiments that were conducted during this study, Fe(II) mediated autotrophic denitrification resulted in fairly high nitrate removal and Fe(II) oxidation efficiencies compared to other denitrification processes. However, several aspects still remain to be

properly assembled and optimized before an efficient configuration at bench-, pilot- and full-scale.

Improvements are especially needed on the investigation of other microorganisms capable for the process. Results showed that all four microbial cultures were capable for the process after longer enrichment in Fe(II). Microorganisms have been isolated from various habitats, such as freshwater and saline environments, paddy soils, ponds, streams, ditches, brackish lagoons, lake sediments, wetlands, aquifers, hydrothermal and deep-sea sediments (Weber et al., 2006; Hedrich et al., 2011) and, therefore, the potential of these microbes is still an aspect that has to be deepened.

The investigation of the operating parameters affecting Fe(II)-mediated autotrophic denitrification indicated that nitrate removal and Fe(II) oxidation are markedly hindered at pH lower than 6. Most of the mining waters and acid mine drainage are extremely acidic. Therefore, a direct application of Fe(II)-driven denitrification to these waters, without preliminary adjusting the pH, can be of high scientific interest.

Despite the current expectations and efforts made in improving the effect of EDTA:Fe(II) ratio, EDTA showed to be inhibitory for denitrifiers. Further investigation of other possible chelating agent should be conducted. NTA (nitrilotriacetic acid) showed to be less inhibitory in nitrate-dependent ferrous oxidizers (Shelobolina et al., 2003). Furthermore, the use of humic substances as chelating agents has been poorly investigated (Kumaraswamy et al. 2006; Larese-Casanova et al., 2010). Another aspect, which was not investigated in this study, is the recycling of EDTA. The use of EDTA results in increasing treatment costs, but EDTA can be effectively recycled and suitable for reuse without losing its chelating properties (Juang and Wang, 2000; Di Palma et al., 2003). An EDTA-recycling step after denitrification can be an interesting option to enhance the economic feasibility of the overall process (Zeng et al., 2005). Design of suitable bioreactor for nitrate removal and Fe(II) oxidation with EDTA-recycling step is an important research perspective.

During this study part of the heavy metals co-precipitates with Fe(III) (hydr)oxides and this can be an important application for the recovery of the products of Fe(II)-mediated autotrophic denitrification. Further research on the effect of single metals and a mixture of them is required, as Fe co-occurs with several metals in real wastewaters.

Moreover, further continuous-flow experiments should be performed in order to reduce the HRT. Then, the operation of bioreactors should be performed with real wastewaters.

Finally, detailed life cycle assessment (LCA) and techno-economic analysis of the whole process, including an environmental impact assessment, need to be performed in order to better evaluate the real efficiency of Fe(II) mediated autotrophic denitrification

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