TAMPEREEN TEKNILLINEN YLIOPISTO
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NITROGEN REMOVAL FROM MINING WASTEWATERS IN BIOREACTORS

Master of Science Thesis

Examiner: Professor Jaakko Puhakka
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PREFACE

This Master's Thesis was conducted as a part of the MINIMAN project, implemented by Tampere University of Technology (TUT), Technical Research Centre of Finland and Geological Survey of Finland. MINIMAN project was aimed at finding solution for nitrogen control in mining activities. TUT's task in the project was to develop high-rate, two-step bioprocess for mine waters based on nitrification and denitrification processes. I started in the project in March 2012 and finished my experiments in October 2012.

I want to thank several people from the Department of Chemistry and Bioengineering from Tampere University of Technology who greatly helped me in the Master's thesis project. First, I want to thank Professor Jaakko Puhakka for giving me this great opportunity to work in the project and to learn a lot. Special thanks belong also to Researcher Stefano Papirio, for his advices, support and encouragement through the project and especially for the corrections in the written report. DFBR experiments, metal toxicity and low pH batch assays were conducted together with him.

Thanks belong also to my collegues Sarita Ahoranta and Antti Venho, Laboratory Technician Tarja Ylijoki-Kaiste and Research Fellows Aino-Maija Lakaniemi and Minna Peltola, who helped me a lot during the project with their positive and supporting attitude and good advices. Thanks also for Emre Oguz Koroglu and Professor Mehmet Cakmakci from Yıldız Technical University for their advices with MBR and for allowing me to use their results from DNMBR experiment in my Master's thesis. I am also grateful to Francesco Di Capua and Researcher Gang Zou, who continued in the MINIMAN project after me and allowed me to use some of the later results from the studied processes. And for the last, but not least, great thanks belong also to my family in Vaasa and my dear friends all over the world.

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ABSTRACT

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One of the main challenges in the mining industry is the emission of harmful components to the environment. Explosive residues and cyanide-containing leaching solutions release ammonium and nitrate to the environment, which can cause eutrophication, formation of nitrous oxide and acid rain. Biological nitrogen removal in municipal wastewater treatment has lower cost and is more efficient than chemical and physical removal methods. Mining wastewaters are usually acidic and contain toxic heavy metals, which can inhibit the biological processes. Therefore, in the present study, the suitability of biological nitrogen removal for mining wastewaters was studied.

Classical biological nitrogen removal generally consists of two processes. Through nitrification ammonium is oxidized to nitrate, which is then reduced to nitrogen gas through denitrification. In this study, two different wastewater treatment plant sludges were used as source of microbial biomass. Main denitrifiers enriched were bacteria belonging to genera *Dechloromonas*, *Rhodobacter* and *Sulphurospirillum*, whereas the main nitrifier found was *Candidatus Nitrospira defluvii.*

Limiting pH for denitrification in batch assays was 4.8. With pH 5.0, denitrification was not inhibited. However, continuous fluidized bed reactors (FBRs) were able to neutralize influent of pH 2.5 due to dilution of influent solution with efficient mixing and produced alkalinity. Denitrification was complete at 7°C and 21°C, within the detection limits, when double stoichiometric ethanol/nitrate ratio was provided. When stoichiometric ratio was provided, some nitrate and nitrite remained.

All studied heavy metals decreased denitrification rate in batch assays. Addition of 50 mg/l of copper, cobalt, nickel and arsenic to batch assays at pH 7 resulted in initial dissolved concentrations of 0.8, 55, 63 and 10.5 mg/l. With these concentrations denitrification rate was 11, 15, 18 and 34% slower, respectively, compared to similar experiments without metals. Added nickel concentration of 100 mg/l resulted in 91% slower denitrification.

Nitrification was efficient in both fluidized bed reactors (FBR) and membrane bioreactors (MBR). Ammonium was completely removed at 21°C when pH was maintained at 6-8.4 with sodiumbicarbonate buffer. Ammonium concentration of 100 mg/l was fed with 7 and 20 h hydraulic retention time (HRT) to FBR and MBR, respectively. In MBR, continuous stirring at 120 rpm provided sufficient sludge suspension and use of aeration directly under hollow fiber membrane prevented membrane fouling.

Simultaneous nitrification and denitrification was investigated in the MBR. Reactor was operated with 60 minutes of aeration and 20-60 minutes of non-aeration intervals. Total nitrogen removal was not as efficient as in separate nitrification and denitrification reactors. The highest total nitrogen removal of 67% was achieved when the non-aeration intervals were 60 minutes. However, for future research, longer enrichment time of the bacteria and further optimization of aeration intervals could enhance nitrogen removal.

In this study, no gaseous denitrification products were analyzed. This would be however essential, since previous results from literature reported increase of N_2O production in low pH and in copper containing studies. It would be also interesting to study metal toxicity in continuous flow reactors. This way cells would have enough time to uptake toxic metals and the long term toxic effects on bacteria could be analysed. In addition, several different metals should be tested at the same time, since mining wastewaters contain multiple metals and their combined effects can be significantly different from effects of one metal at a time.

TIIVISTELMÄ

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Kaivosteollisuuden merkitys maailman taloudelle on huomattava, sillä monet teollisuuden alat perustuvat kaivosteollisuuden tuottamien raaka-aineiden varaan. Kaivosteollisuus työllistää huomattavan määrän työntekijöitä vaikuttaen näin yksittäisten valtioiden paikalliseen talouteen. Yksi kaivosteollisuuden isoimmista haasteista on kuitenkin kaivoksien ympäristövaikutusten vähentäminen. Typpeä sisältävien räjähteiden ylijäämät ja metallien liuotuksessa syntyvät syanidipitoiset liuokset vapauttavat ammonium ioneja ja nitraattia kulkeutuessaan ympäristöön aiheuttaen siten ympäröivien vesistöjen rehevöitymistä, dityppioksidipäästöjä ja happamia sateita.

Typpipäästöjä voidaan vähentää käsittelemällä kaivoksien jätevesiä joko kemiallisesti, fysikaaliset tai biologisesti. Biologinen typenpoisto on osoittautunut näistä menetelmistä edullisimmaksi ja tehokkaimmaksi. Kaivoksien jätevedet ovat kuitenkin yleensä happamia ja sisältävät myrkyllisiä raskasmetalleja, jotka voivat hidastaa tai jopa estää kokonaan biologisien prosessien toiminnan. Tässä tutkimuksessa tutkittiin biologisen typenpoiston soveltuvuutta typen poistoon kaivoksien jätevesistä.

Perinteinen biologinen typenpoisto koostuu kahdesta erillisestä prosessista. Ammonium typpi muutetaan ensin nitrifikaatiossa nitraatiksi ja nitraatti edelleen denitrifikaatiossa typpikaasuksi. Nämä kaksi prosessia eroavat toisistaan huomattavasti. Nitrifikaatio on aerobinen prosessi, jossa mikrobit hapettavat epäorgaanisia typpiyhdisteitä tuottaakseen energiaa solun toimintoja varten. Solujen kasvua varten tarvittavat hiiliyhdisteet saadaan pelkistämällä hiilidioksidia ilmasta. Denitrifikaatio puolestaan on anaerobinen prosessi, jonka avulla solu hankkii energiaa hapettamalla epäorgaanisia tai orgaanisia hiiliyhdisteitä ja pelkistämällä nitraattia ja nitriittiä.

Tässä tutkimuksesta mikrobiyhteisö rikastettiin jäteveden puhdistamon aktiivilieteprosessista. Tärkeimpiä denitrifikaatioon osallistuvia mikrobeja olivat *Dechloromonas*, *Rhodobacter* ja *Sulphurospirillum* sukuihin kuuluvat bakteerit. Yleisin nitrifioiva mikrobi oli *Candidatus Nitrospira defluvii.*

Tutkimuksessa havaittiin seuraavat denitrifikaatiota rajoittavat tekijät. Panoskokeissa pH 4.8 esti denitrifikaation kokonaan. Kuitenkin, syöteliuoksen matalalla pH:lla (jopa pH 2.5) ei ollut vaikutusta denitrifikaatioon jatkuvasyötteisiä leijupetireaktoreita käytettäessä. Denitrifikaatiossa muodostuvat emäksiset yhdisteet neutraloivat syötteen reaktorissa nopeasti tehokkaan sekoituksen ansiosta. Nitraatin ja nitriitin pelkistys oli 100 % mittaustarkkuuden rajoissa, sekä 7°C että 21°C lämpötiloissa, kun syötteeseen lisättiin etanolia hiilenlähteeksi kaksi kertaa enemmän kuin stoikiometrisesti laskettuna

olisi ollut tarpeellista. Lisättäessä vain stoikiometrinen määrä etanolia, osa nitraatista ja nitriitistä jäi pelkistymättä.

Kaikki tutkitut raskasmetallit hidastivat denitrifikaatiota panoskokeissa. Kun kuparia, kobolttia, nikkeliä ja arseenia lisättiin 50 mg/l, niiden liukoiset pitoisuudet kokeen alussa olivat 0.8, 55, 63 ja 10.5 mg/l. Näillä pitoisuuksilla denitrifikaatio oli 11, 15, 18 ja 34 % hitaampaa kuin panoskokeissa ilman metalleja. Suurempi lisätty nikkelipitoisuus, 100 mg/l, hidasti denitrifikaatiota 91%. Metallien haitallisuus denitrifikaatiolle lisääntyi seuraavassa järjestyksessä: Cu < Co < Ni < As.

Nitrifikaatio oli tehokasta sekä leijupeti- että kalvoreaktoreissa. Ammoniumtyppi saatiin täysin hapetettua 21°C lämpötilassa, kun pH ylläpidettiin välillä 6-8.4 natriumbikarbonaattipuskurin avulla. Ammoniumtyppeä pumpattiin näihin reaktoreihin 100 mg/l, seitsemän ja kahdenkymmenen tunnin hydraulisilla viipymillä. Kalvoreaktoria käytettäessä jatkuva sekoitus 120 rpm -vauhdilla takasi riittävän biomassan sekoittumisen ja ilmastuksen asentaminen suoraan kalvon alle esti kalvon tukkeutumisen.

Nitrifikaation ja denitrifikaation yhdistämistä samaan kalvoreaktoriin tutkittiin. Ilmastus käynnistettiin 60 minuutiksi ja kytkettiin pois päältä 20-60 minuutiksi, mahdollistaen sekä hapelliset olosuhteet nitrifikaatiolle, että hapettomat denitrifikaatiolle. Paras kokonaistypen poisto, 67%, saavutetiin kun ilmastuksen poiskytkentäaika oli 60 minuuttia.

Tässä tutkimuksessa denitrifikaation kaasumaisia tuotteita ei analysoitu. Se olisi kuitenkin tärkeää, sillä matalan pH:n ja raskasmetallien on raportoitu lisäävän haitallisia dityppioksidipäästöjä, turvallisen typpikaasun sijaan. Lisäksi olisi mielenkiintoista tutkia myös raskasmetallien vaikutuksia jatkuvasyötteisissä reaktoreissa. Panoskokeissa metallien pitoisuus solujen sisällä on aluksi niin vähäinen, että metallien todelliset pitkäaikaiset vaikutukset saattavat jäädä huomaamatta. Myös monien metallien yhteisvaikutukset tulisi tutkia, sillä ne saattavat poiketa huomattavasti yhden metallin vaikutuksista.

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ABBREVIATIONS

AAS	Atomic absorption spectrophotometer
AMO	Ammonium monoxygenase
Anammox	Anaerobic ammonium oxidation
ANFO	Ammonium nitrate fuel oil
ATP	Adenosine triphosphate
Cyt c	Cytochrome c
DFBR	Denitrification fluidized bed reactor
DFBR1	Denitrification fluidized bed reactor 1, at 7°C
DFBR2	Denitrification fluidized bed reactor 2, at 21°C
DFBR3	Denitrification fluidized bed reactor 3, at 21°C, used as biomass source for batch bottle experiments
DGGE	Denaturing gradient gel electrophoresis
DMBR	Denitrification membrane bioreactor
DNMBR	Simultaneous denitrification and nitrification in membrane bioreactor
DO	Dissolved oxygen
EPS	Extracellular polymeric substances
FBR	Fluidized bed reactor (In this study: up-flow fluidized bed reactor)
HAO	Hydroxylamine oxidoreductase
HPLC	High pressure liquid chromatography
HRT	Hydraulic retention time
IC	Ion chromatography
MBR	Membrane bioreactor (In this study: Membrane filtration bioreactor)
mQ	Water filtrated and deionized with Milli-Q Plus machine and M-millipore QPAK®1 filtrate (Millipore, USA). Water resistivity 18.2 mΩ
NFBR	Nitrification fluidized bed reactor (NFBR)
NMBR	Nitrification membrane bioreactor
NXR	Nitrite oxidoreductase
PCR	Polymerase chain reaction
Q	Ubiquinone
SMP	Soluble microbial products
SRT	Sludge retention time
TUT	Tampere University of Technology
VSS	Volatile suspended solids

1. INTRODUCTION

Mining industry has great impact on global economy. Different mining products are needed in majority of industrial fields including construction of buildings, roads and power plants and production of fertilizers, machines and electronics. It is estimated that half of world's mineral production is localized in politically unstable areas. For example, the European Union consumes 30% of mining products, but it only produces 3 %. European commission has listed all the metals that are critical or economically important, but have uncertain availability in the future. Among these metals are niobium, cobalt, nickel, chromium, iron, manganese, vanadium, zinc, copper and titanium, which are either at the production in Finland or have good discovery potentials. Mining industry in Finland is also important for regional employment. Together with natural stone and aggregate industry, mining industry employs around 12000 persons with annual turnover of 1700 million euros. It is estimated that each employee at mining industry creates other three or four jobs, increasing significance of the employment. (GTK 2012).

One of the main challenges in the mining industry is the emission of harmful components to the environment (GTK 2012). The present study concentrates on nitrogen removal methods from mining wastewaters. Mines are not the major nitrogen pollution sources for the ecosystem, but have significant impacts on the environment locally. Nitrogen compounds such as nitrate and ammonium are highly soluble and easily transferred along drainages to the environment (Almasri 2007; Morin & Hutt 2009). Excess of nitrate compounds in drinking water can cause toxic effects on human health (WHO 2011), for example, methemoglobinemia to infants and stomach cancer to adults (Lee 1992, cited in Almasri 2007). Too high nitrate levels in aquatic environment cause eutrophication and formation of nitrous oxide (N_2O), a powerful greenhouse gas (Hu et al. 2012). Sunlight can further convert N_2O to NO, which reacts in the atmosphere with ozone (O_3) forming nitrite (NO_2^-). Nitrite produces nitrous acid (HNO_2) with moisture and rains back to grounds as acidic rain. (Madigan et al. 2008).

Nitrogen compounds are degraded in the environment with various natural processes. Figure 1 demonstrates natural nitrogen compound degradation and transformation in soils. Ammonium can be used for production of organic matter (immobilization) or converted to nitrate (nitrification). If nitrate is not further converted to nitrogen gas through denitrification, it eventually drifts to groundwater. (Almasri 2007). Similar processes also occur in other aquatic environments. Natural denitrification in Gulf of Finland removes annually 30 % of nitrogen load (Tuominen et al. 1998). Other reported natural denitrification percentages are 50-80 % (Seitzinger et al. 2006, cited in

Magalhaes et al. 2011). However, remarkable negative impacts of nitrogen in water systems and uncertain removal efficiency in nature create needs to limit nitrogen discharges to the environment. During mining activities, there are two possibilities to reduce nitrogen release. Main nitrogen sources, such as explosive residues and cyanide leaching by-products, can be reduced by process optimization. Other option is nitrogen removal from the wastewaters produced. Biological nitrogen removal has shown to be efficient and economical compared to chemichal and physical processes (Siegrist 1996).

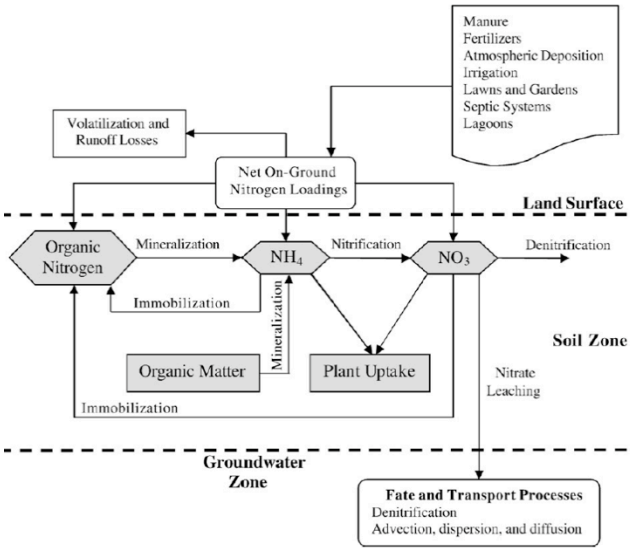

Figure 1. Demonstration of different nitrogen sources, transformation processes and movements in land, soil and groundwater. (Almasri 2007).

Biological nitrogen removal is limited by several characteristics of mining wastewaters, such as low temperature, low pH and dissolved toxic heavy metals and metalloids. In this study, the following parameters affecting the treatment of nitrogen-contaminated mine waters were studied:

1. Effect of pH, copper, cobalt, nickel and arsenic on nitrate removal in batch assays.
2. Effect of pH and cold temperature on nitrate removal in continuous flow fluidized bed reactor.
3. Suitability of fluidized bed and membrane bioreactors for nitrification
4. Simultaneous nitrification and denitrification in a single continuous flow membrane bioreactor.
5. Characterization of microbial communities in biological reactor processes, identified with 16S RNA sequencing method.

2. THEORETICAL BACKGROUND

2.1. Mines and mining wastewater characteristics in Finland

History of Finnish mining industry has been comprehensively gathered by Puustinen (2003). Kaksonen (2004) has reviewed mining industry magnitude in Finland at 2004. Iron ore mining was started in 1540s. Together 418 metal ore and 616 industrial mineral and carbonates stone mines have existed between 1533 and 2001. (Puustinen 2003). Figure 2.1.1.a presents historical and operating metal ore mines in Finland until 2004. Figure 2.1.1.b presents vision of operating mines at 2010 for year 2020 from Finland's Mineral strategy (GTK 2012), indicating that mining industry has potential in Finland also in the future.

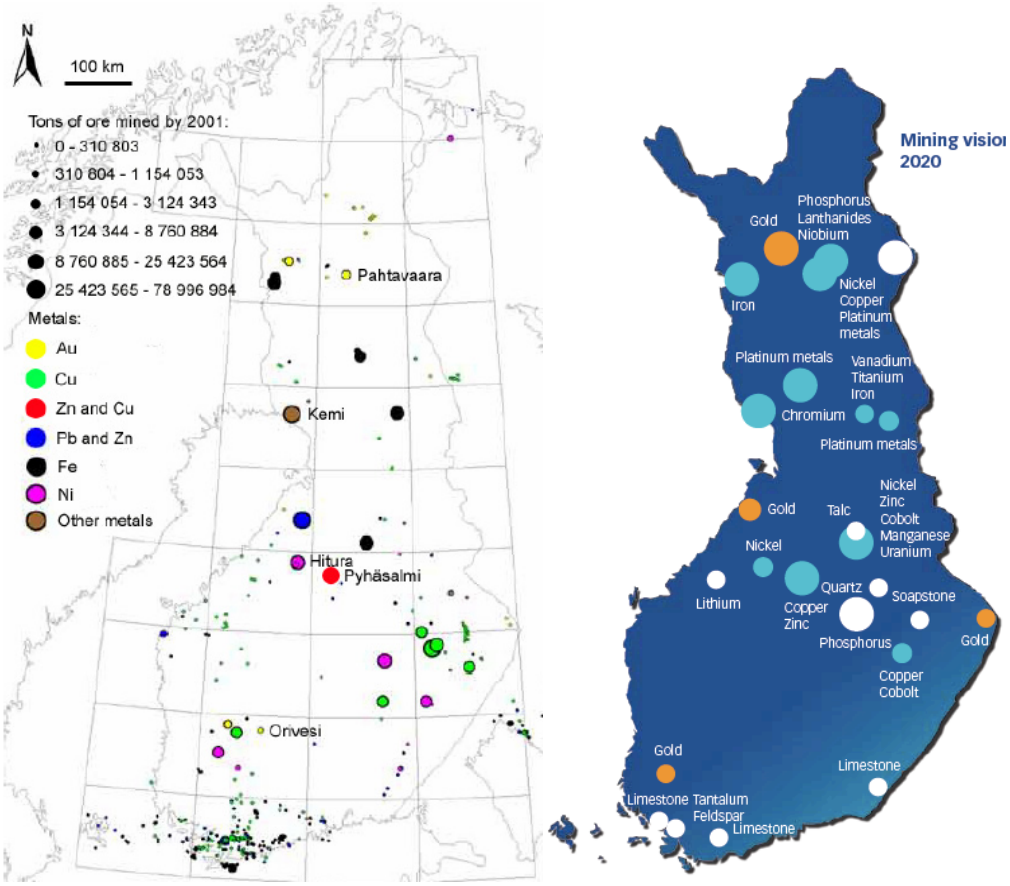

Figure 2.1.1 a) Tons of ore mined in Finland 1553-2001 and operating mines 2004 (Kaksonen 2004), adapted from (Puustinen 2003). b) Finnish mining vision 2020 (GTK 2012)

Kaksonen (2004) has reviewed acid mine drainage formation in mines and its effects on the environment. When mining exposes metal sulphides to oxygen and water, they are oxidized and dissolved in the water producing metal ions, sulphate and acidity. In addition free metal ions hydrolyse with water further producing acidity. As examples, overall reactions of pyrite (FeS_2) oxidation and aluminium (Al_3^+) hydrolysis are presented in the equations 2.1.1 and 2.1.2. (EPA 1994a; Banks et al. 1997, cited in Kaksonen 2004)

$$4FeS_2 + 14H_2O + 15O_2 \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+ \quad (2.1.1)$$

$$Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+ \quad (2.1.2)$$

2.2. Nitrogen in mining waters

Main nitrogen sources in the mining sites are explosive residues and compounds from cyanide destruction in cyanide metal leaching (Morin & Hutt 2009; Chlot 2011). Other sources are transformation of amines in flotation circuits, regulating agents for pH control, ammonium sulphate and ammonium hydroxide used in uranium treatments and ammonium used for copper and nickel hydrometallurgical processes (EPA 2003, cited in Mattila et al. 2007).

2.2.1. Nitrogen from explosive residues

Equation 2.2.1.1 shows the explosion reaction of ammonium nitrate fuel oil (ANFO) explosives. If blasting is not complete, some ammonium nitrate (NH_4NO_3) remains in the mining sites. Fuel oil in the equation 2.2.1.1 is marked as CH_2. (Morin & Hutt 2009)

$$3NH_4NO_3 + CH_2 \rightarrow 7H_2O + CO_2 + 3N_2 + heat\ (912\ kJ/mol) \quad (2.2.1.1)$$

Factors affecting blasting efficiency are drilling, packing of the explosives in the holes, detonation sequences and reliability. Approximately, 5-15 % of total ANFO is not detonated, but leached in the mines (Wiber et al. 1991; Sharpe 2007, cited in Morin & Hutt 2009). With proper handling, leaching could be reduced to 2-5 % (Sharpe 2007, cited in Morin & Hutt 2009).

Research study from Canadian open pit and underground mines (Ferguson & Leask 1988, cited in Morin & Hutt 2009) indicated that in dry conditions lost of nitrogen could be only 0.2 %, lower than in wetter conditions (2-5 %). This is due to ANFO dissolution (Wiber et al. 1991, cited in Morin & Hutt 2009). Relative nitrogen compound discharge concentrations in the Canadian study (1988) were 51-56 % nitrate, 40-46 % ammonium and 3-4 % nitrite. Nitrogen discharges from mines to their nearest aquatic environments in study of Forsberg & Åkerlund (1999, cited in Mattila, et al. 2007) were approximately 0.03-1.0 % (w/w). Estimated annual consumption of explosives in

Finland at 2006 was 12 000 tons, calculated by extracted rock quantity of 30 600 000 tons and explosive consumption of 0.4 kg explosives / ton of rock. This results in 36-120 tons of nitrogen discharge annually to mining wastewaters in Finland.

2.2.2. Nitrogen from cyanide leaching process

Cyanide is used in mines to extract gold and other valuable metals from ores. Sodium or potassium cyanides react with metal ores forming dissolved compounds. After leaching, cyanide is destructed. (Akcil 2002). Different destruction methods are sulphur oxidation, alkaline chlorination, air oxidation, use of hydrogen peroxides and biological treatment, described in the equation 2.2.2.1. Cyanide destruction produces cyanates (CNO^-) that are less toxic than cyanides and further oxidized to ammonia and bicarbonates as described in equation 2.2.2.2. (EPA 1994b)

$$Cu_2CN + 2H_2O + \tfrac{1}{2}O_2 \rightarrow Cu(biofilm) + HCO_3 + NH_3 \qquad (2.2.2.1)$$

$$CNO^- + 2H_2O \rightarrow CO_3^{2-} + NH_3 \qquad (2.2.2.2)$$

Several natural cyanide degradation processes are listed in EPA 1(994b). Among these methods, microbial generation of ammonium and anaerobic biodegradation with H_2S produce nitrogen compounds.

2.3. Biological nitrogen removal

Biological nitrogen removal is widely used in the industrial and municipal wastewater treatment plants. Tchobanoglous & Burton (1991) and Siegrist (1996) revealed that biological nitrogen removal is the most economical method compared to traditional physical and chemical ones, such as air stripping, breakpoint chlorination and ion exchange.

There are two different kinds of natural biological nitrogen removal processes. In classical process ammonium is first converted to nitrate in aerobic oxidation process called nitrification. Nitrate is then further converted to nitrogen gas in anaerobic respiration process called denitrification. (Rittman & McCarty 2001; Madigan et al. 2008). Ammonium oxidation and nitrate reduction can be even combined in one single process called anaerobic ammonium oxidation (anammox) (van de Graaf et al. 1996; Egli et al. 2001; Zhang et al.2010; Chen et al. 2011). Partial nitrification converts part of the ammonium to nitrite, which is further used as an electron acceptor, with the remaining nitrate in solution, in anoxic ammonium oxidation (Madigan et al. 2008). These two processes are presented in Figure 2.3.1.

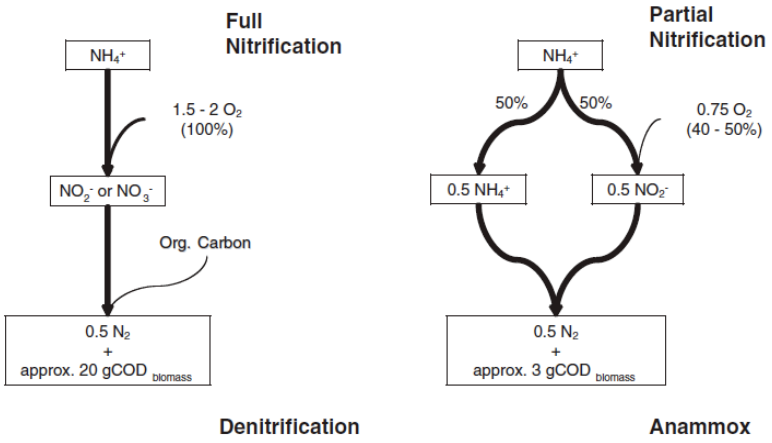

Figure 2.3.1 Two methods for biological ammonium (NH_4^+) removal: a) classical nitrification with denitrification and b) partial nitrification with anammox (Fux & Siegrist 2004).

Fux & Siegrist (2004) compared the two processes presented in Figure 2.3.1. Partial nitrification and anammox resulted to be cheaper, since organic electron donors and a strong aeration are not required. Moreover, biomass yield is low and no harmful nitrous oxide is produced (van de Graaf et al. 1996). Thus, the anammox would suit especially for wastewaters with low carbon to nitrogen ratio, including mining effluents. However, anammox bacteria are slowly growing organisms and their enrichment takes much more time than the classical nitrifying/denitrifying bacteria Doubling times of 11-30 days are reported for anammox microorganisms (Strous et al. 1998; van Niftrik et al. 2004). In addition, many studies indicate optimal temperatures of 30-37°C (Egli et al. 2001; Fux & Siegrist 2004), and limiting temperatures of 20-22°C (Isaka et al. 2007; Yang et al. 2010). In most areas of Finland, the average temperature range is between 1 and 6 °C and, even in the summer, temperature stays below 25°C (Ilmatieteen laitos 2012). Use of anammox process in Finland might thus require heating, resulting in extra treatment costs. Anammox process has also shown optimal pH around 8.0 (Egli et al. 2001) and inhibition already at pH 6.5 (Egli et al. 2001; Zhang et al. 2010) probably due to accumulation of free nitric acid. In this study, biological nitrogen removal, through classical nitrification and denitrification, was chosen. Nitrogen removal was studied in separate and combined nitrification and denitrification reactors.

2.3.1. Nitrification

Nitrification process is aerobic and divided in two steps. Ammonium is first oxidized to nitrite as presented in the equation 2.3.1.1. Nitrite is then further oxidized to nitrate according to equation 2.3.1.2. (Madigan et al. 2008; Tchobanoglous & Burton 1991)

$$NH_4^+ + \tfrac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad (2.3.1.1)$$

$$NO_2^- + \tfrac{1}{2}O_2 \rightarrow NO_3^- \qquad (2.3.1.2)$$

Overall reaction for nitrification is described in the equation 2.3.1.3. It shows that nitrification produces protons decreasing the pH.

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \qquad (2.3.1.3)$$

2.3.1.1 Biochemistry

Ammonium and nitrite are reduced inorganic compounds. As they are oxidized in nitrification, their electrons enter the electron transport chain in the cell membrane. Electron transport chain produces a proton motive force, an electrochemical potential in the cell membrane, which transfers protons from cell cytoplasm to periplasm. Return of protons to cytoplasm through *ATP synthase* enzyme provides energy needed to produce Adenosine triphosphate (ATP) for the cell (Madigan et al. 2008).

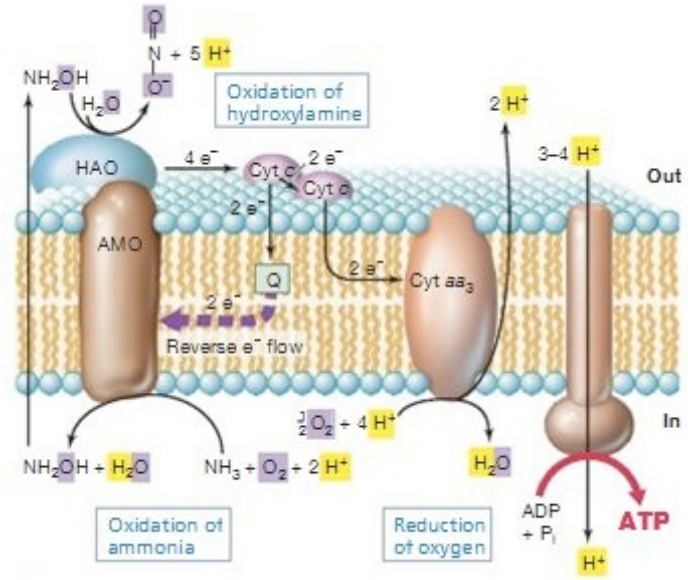

Figure 2.3.1.1.1. Ammonium oxidation to nitrite. (Madigan et al. 2010, Figure 13.26)

Figure 2.3.1.1.1 displays the ammonium oxidation to nitrite. *Ammonium monooxygenase* (AMO) is a protein inside the membrane that catalyses ammonia (NH_3) oxidation to hydroxylamine (NH_2OH). *Hydroxylamine oxidoreductase* (HAO), a periplasmic protein outside the membrane, oxidizes NH_2OH further to nitrite (NO_2^-) producing four electrons. Two of the electrons transferred to cytochrome *c* (Cyt c) and ubiquinone (Q) for energy needed in the ammonium oxidation, yielding only two produced electrons for the energy production in cytochromes *aa3*. (Madigan et al. 2008).

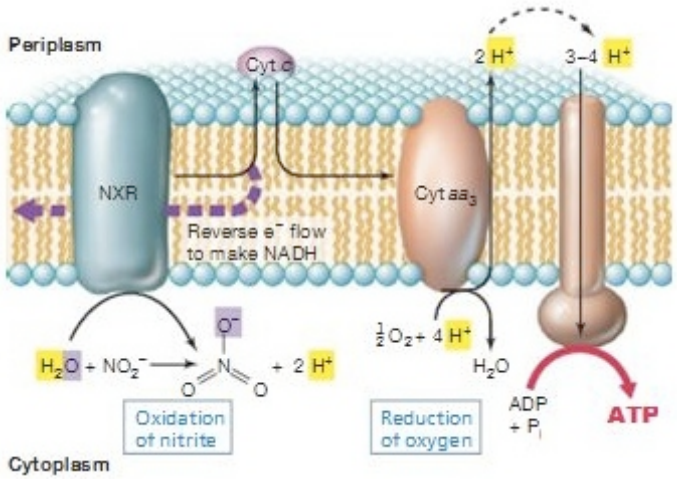

Figure 2.3.1.1.2. Nitrite oxidation to nitrate. (Madigan et al. 2010, Figure 13.27)

Figure 2.3.1.1.2 displays nitrite oxidation to nitrate. *Nitrite oxidoreductase* (NXR) enzyme in the cell membrane oxidizes nitrate to nitre producing electrons that are transfer to aa_3 through Cyt c. (Madigan et al. 2008).

2.3.1.2 Microorganisms

Nitrifying bacteria use inorganic nitrogen in chemolitrophic growth. They can be found from any of the four *Proteobacteria* classes: *Alpha, Beta, Gamma* and *Delta. Nitrospira* genus belongs to its own phylym. Nitrifying bacteria can be further divided to ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, according to their oxidation activity (Madigan et al. 2008). *Nitrosomonas* is the most common genus of bacteria in ammonium oxidation and *Nitrobacter* and *Nitrospira* in nitrite oxidation. (Teske et al. 1994). In addition to bacteria, an autotrophic ammonia-oxidizing chemolitotrophic *Archaea,* called *Nitrosopumilus,* exists. (Madigan et al. 2008).

Nitrifying bacteria are autotrophs and obligate aerobes. They use inorganic nitrogen compounds as electron donors and fix and reduce inorganic carbon with Calvin cycle for their cell growth. (Madigan et al. 2008). They do not need external carbon source, but their growth rate is relatively slow. As obligate aerobes, they need oxygen as terminal electron acceptor in the respiration. If dissolved oxygen (DO) concentration is too low, reaction is inhibited. (Rittman & McCarty 2001).

2.3.2. Denitrification

Denitrification is an anaerobic respiration process, where nitrate and nitrite are used as electron acceptors. Denitrification can be carried out both by heterotrophic and autotrophic microorganisms. Heterotrophic denitrification is more studied and widely used in wastewater treatment. (Park & Yoo 2009).

In the present study, ethanol was used as electron donor/carbon source. Equation 2.3.2.1 described denitrification using ethanol. One of the products of the reaction is

alkalinity that increases the solution pH. (Rittman & McCarty 2001; Madigan et al. 2008).

$$12NO_3^- + 5CH_3CH_2OH \rightarrow 6N_2 + 10CO_2 + 9H_2O + 12OH^- \qquad (2.3.2.1)$$

2.3.2.1 Biochemistry

Reduction of nitrate and nitrite consists of several steps catalysed by different enzymes. Figure 2.3.2.1.1 shows an example of denitrification in membrane of *Pseudomonas stutzeri*. *Nitrate reductase*, a membrane protein, converts nitrate (NO_3^-) to nitrite (NO_2^-). Then *nitrite reductase,* a periplasmic protein, converts nitrite to nitric oxide (NO). Nitric oxide is further oxidized to nitrous oxide (N_2O) by *nitric oxide reductase*. Finally nitrous oxide is oxidized to nitrogen gas (N_2) by *nitrous oxide reductase*. These enzymes are repressed by presence of molecular oxygen O_2 or by lack of nitrate. (Madigan et al. 2008). Nitrite reductase and nitrous oxide reductase are more sensitive to oxygen than nitrate reductase, resulting in an accumulation of the intermediates. (Rittman & McCarty 2001).

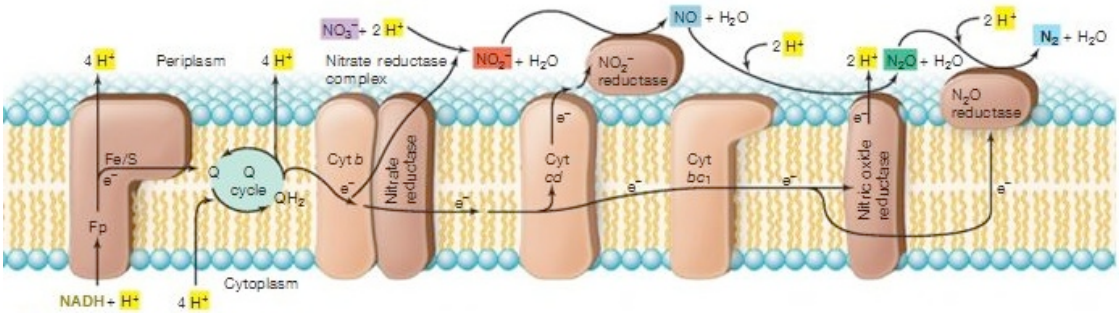

Figure 2.3.2.1.1. Enzymes involved in nitrate conversion to nitrogen gas in the cell membrane (Madigan et al. 2010)

2.3.2.2 Microorganisms

Denitrifiers are common in sediments, soils, ground waters, surface waters and wastewater treatment plants. Most denitrifying microorganisms belong to *Proteobacteria.* These are genera such as *Pseudomonas*, *Alcaligenes*, *Paracoccus* and *Thiobacillus*. They are facultative anaerobes indicating that, if free oxygen is present, aerobic respiration occurs instead of denitrification. Many of them can also use other electron acceptors like ferric ion (Fe^{3+}) in anaerobic respiration. Some bacteria from genus *Bacillus*, some halophilic archaea and amoeba eukaryote *Globulimina pseudospinescens* are also able to denitrify. (Rittman & McCarty 2001; Madigan et al. 2008).

2.3.2.3 Electron donors

External carbon sources are needed for denitrification if organic content in wastewater is very low. Inorganic electron donors, such as hydrogen gas H_2 (g) or reduced elemental sulphur S (s), can be used for autotrophic denitrification. Biomass yield is

lower in the heterotrophic growth. However, the utilization and supply of these substrates is not simple to manage. Hydrogen gas causes safety problems in transfer systems and sulphur oxidation produces strong acids. (Rittman & McCarty 2001; Park & Yoo 2009)

Organic electron donors favour heterotrophic denitrifiers and are commonly used since they are easily available. Methanol, ethanol, acetate and methane have been compared in various studies. Results vary depending on used operating conditions and microorganisms. Traditionally, methanol has been used because of low price. Ethanol has however shown similar and better results compared to other organic carbon sources (Santos et al. 2004; Adav et al. 2010). Borden et al. (2011) reported successful studies using ethanol and other organic carbon sources as electron donors to enhance denitrification in laboratory and field-scale (Tartakovsky et al. 2002; Santos et al. 2004; Adav et al. 2010).

The concentration of added external electron donor should be carefully calculated. Electron donor supply is usually the main expense that has to be highly taken into account especially in the industrial scale. The excess of organic electron donor may results in undesired organic rest pollution. On the contrary, too small concentrations can limit the reaction rates (Rittman & McCarty 2001). For instance, nitrite reductase enzyme has been shown to be more sensitive to electron deficiency than nitrate reductase. This can lead to nitrite accumulation (Neubauer & Gotz 1996) and inhibition of nitrate, nitrite and nitrous oxide reducatases (Almeida et al. 1995, p.476; Adav et al. 2010). Therefore, the addition of a proper amount of electron donors increases the activity of nitrite reductase resulting in higher denitrification rates (Adav et al. 2010).

Electron donor consumption per certain nitrate concentration can be estimated from stoichiometric denitrification equation. For example equation 2.3.2.1 has been balanced using ethanol as electron donor, each 12 moles of nitrate consumes stoichiomterically 5 moles of ethanol. In addition, ethanol utilization by competing microorganisms and for biomass growth has to be considered as well.

2.3.3. Effects of low temperature

Denitrification has shown to proceed at low temperatures. Complete denitrification is reported at 6±2°C pilot scale soil reactors (Martin et al. 2009) and 5°C immobilized bioreactors. However, decrease in temperature from 30 to 5°C has shown significant decrease of denitrification rate (Stanford et al. 1975; Fischer & Whalen 2005; Vackova et al. 2011).

Saleh-Lakha et al. (2009) studied the effect of temperature on denitrification with pure culture of *Pseudomonas mandelii*. Decrease of temperature from 30 to 10 °C did not inhibit the expression of nitrite and nitrite oxide reductase genes, but their expression was delayed (Saleh-Lakha et al. 2009).

2.3.4. Effects of low pH

Low pH has two effects on denitrification. It inhibites total denitrification and increases production of harmful nitrous oxide.

According to Knowles (1982), optimal pH was between 7 and 8. EPA (1975) reported an optimal pH range of 6-8. Inhibition at low pH results in nitrite and its protonated form, nitrous acid (pK_a=3.7) accumulation (Baeseman et al. 2006). Nitrous acid has shown to inhibit denitrification already at concentrations as low as 0.04 mg HNO_2-N /l. (Abeling & Seyfried 1992, cited in Baeseman et al. 2006).

Accumulation of nitrous oxide results from the competition for electron donors between different nitrogen reducing enzymes. Pan et al. (2012) oberved that at pH 6.0 20 and 40 % of nitrogen accumulated as N_2O, with and without added external methanol, respectively. At pH 6.5, HNO_2 accumulation percentages were 0 and 30%, whereas no nitrous oxide was formed in the pH range 7.0-9.0. Thörn & Sörensson (1996) report following N_2O formation percentages: 100% at pH below 5, 40% at pH 6.0 and 0% at pH above 6.8.

2.3.5. Effects of heavy metals

Biological function of heavy metals is based on their incompletely filled d orbitals, which are able to form complex compounds. Some heavy metals have important function in the cells as trace metals (Zn, Ni, Co). Because of that, cells have developed two different mechanisms to uptake metals. The first one is called chemiosmotic gradient and it transfers metals passively through the cell membrane. It is the faster method and it cannot be specifically regulated. The second mechanism is the specific transfer. It is slower, uses energy and is induced only when metals are needed in the cell. (Nies 1999). Most of times, metal uptake is toxic for cell function. Cell enzymes and membrane receptors are very sensitive to complex forming metals (Klaassen 2001). Uptaking of metals makes cells more vulnerable. Thus, due to the common presence of harmful metals in the environment, microorganisms have developed metal resistant features presented in Figure 2.3.5.1. Cells can be protected from toxic effects of metals by reducing metal permeability and increasing metal sequestration, active transport via efflux pumps and enzymatic detoxification. (Liu et al. 2001; Tanaka et al. 2004b, cited in Principi et al. 2006). In addition, increased production of extracellular polymeric substances (EPS) enhances metal resistance by binding heavy metals outside the cells and retarding their accumulation (Lawrence et al. 2004). Inside the cells, thiol ligands of *metallothionein* proteins bind to many toxic metals (Cd, Zn, Co, Hg, Ag, Cu) reducing their toxicity. (Klaassen 2001)

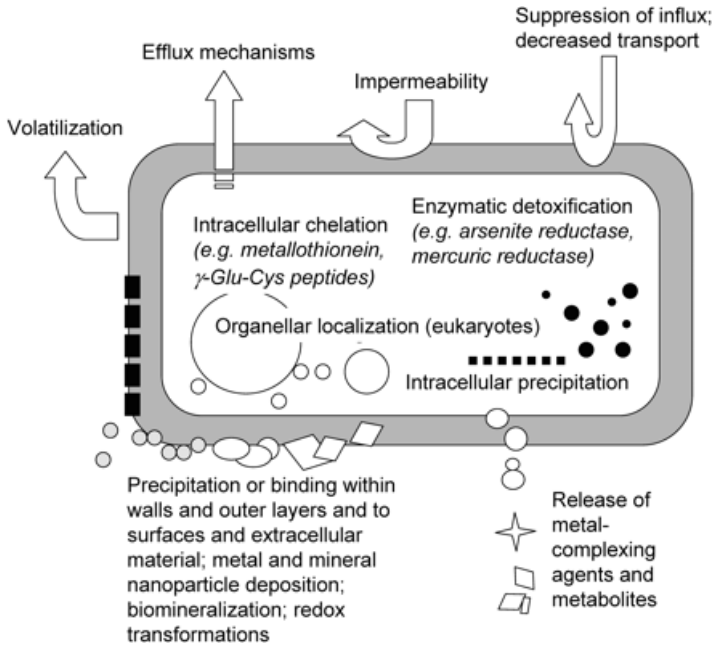

Figure 2.3.5.1. Possible metal resistant features of microorganisms (Gadd 2009, cited in Gadd 2010).

Nitrifying bacteria has shown ability to adapt into metal containing environments. For example, nickel tolerance has shown to increase in the laboratory during 28 days incubation and better copper resistance has been found in nitrifiers exposed to copper for 80 years, compared to non-exposed cultures. (Fait et al. 2006)

Different metal toxicity studies from literature are not directly comparable due to differences in the operating conditions. Continuous reactor experiment showed higher metal toxicity of Cu^{2+}, Zn^{2+}, $Ni^{2+}$and Cd^{2+} on nitrification than similar batch tests (Hu et al. 2004). This was supported by results indicating that inhibition of Zn^{2+}, $Ni^{2+}$and Cd^{2+} was caused by their continuous accumulation inside the cells (Hu et al. 2003).

2.3.5.1 Nickel

Nickel affects the DNA function by replacing Zn^{2+} ions with Ni^{2+}, as they have similar ionic radius. (Klaassen 2001). In addition, nickel has been reported to reduce EPS formation in biofilms. (Lawrence et al. 2004).

2.3.5.2 Copper

Copper has diverse effects on nitrifying and denitrifying bacteria. It is an essential micronutrient in small quantities (6 pmol/l, Granger & Ward 2003) and necessary constituent for certain reductase enzymes (Zumft 1997).

Toxicity of copper results from two reaction mechanisms. Firstly, an excess of copper catalyzes production of hydroxyl radicals and causes disruption of membrane functions. (Howlett & Avery 1997, cited in Hu et al. 2003; Nies 1999). Secondly, dissolved copper ions have high affinity to form complexes with extracellular polymeric substances (EPS) (Nies 1999; Liu et al. 2001, cited in Cecen et al. 2009). Hu et al. (2003) observed that the inhibitory mechanisms of copper did not derive from metal

accumulation inside the cell. In addition, copper does not induce metallothionein production efficiently, unlike zinc or cadmium, increasing its toxic effects (Klaassen 2001).

2.3.5.3 Arsenic

Arsenic exists in two major forms: as trivalent As(III) in arsenite (AsO_2^-) and as pentavalent As(V) in arsenate (AsO_3^{4-}). Arsenite is uptaken and removed from cells by simple diffusion (Huang & Lee 1996; Nies 1999). Arsenate structure is very similar to phosphate, permitting As(V) to enter to cell via phosphate transportation systems (Nies 1999; Klaassen 2001). Thus, to be removed from the cells, arsenate has to be reduced to arsenite, separating it from the phosphates. (Nies 1999).

Arsenite is regarded as the most toxic form of arsenic, affecting cell function in many ways. For example, it competes with phosphate in oxidative phosphorylation, decreasing ATP formation. In addition higher hydrogen peroxide concentration increases oxidative stress. (Klaassen 2001). Possible protein-DNA crosslinks prevent DNA replication and methylated forms cause inhibition of pyryvate dehydrogenase, essential enzymes for the cellular respiration. (NRC 2001).

2.3.5.4 Cobalt

Cobalt is needed in B_{12} co-factors. It is transported into the cells via chemiosmotic gradient. (Nies 1999).

2.3.6. Simultaneous nitrification and denitrification in a single reactor

Figure 2.3.6.1 demostrates different configuration possibilities for total nitrogen removal in bioreactors. As classical nitrification and denitrification require different oxygen levels, it is necessary to separate the two processes either spatially or temporally (Li et al. 2008). The processes can be developed in two distinct reactors or combined in a single reactor. The first configuration is more expensive but results in an easier control of the process. The second configuration requires more attention, especially in terms of oxygen concentration, but the smaller volumes result in lower costs (Li et al. 2008).

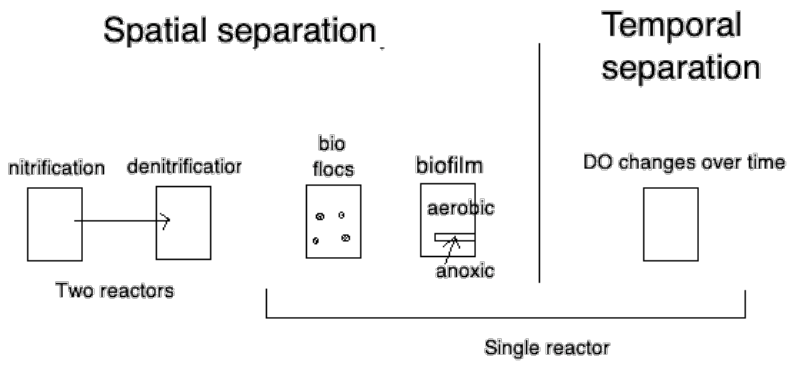

Figure 2.3.6.1. Possible configurations for the separation of nitrification from denitrification

Using a temporal separation configuration, oxygen level in the solution varies over time. With this method, sufficient oxidation supply for nitrification and enough low DO concentration for denitrification are essential (Wang et al. 2012). Wang et al. (2012) used a four-step process, where oxic and anoxic zones changed repeatedly, gaining 86.4% and 95.7% total nitrogen and ammonium removal, respectively, from urban wastewaters.

The separation of nitrification from denitrification can be performed spatially, even in a single reactor, if anoxic zones are formed. Pochana & Keller (1999, cited in Li et al. 2008) reported anoxic gradient formation in bioflocs of size 50-110 nm. When flocs were smaller, oxygen passed through inhibiting denitrification. Holman & Wareham (2005) exploited anoxic microbial flocs, achieving total nitrogen removal of 75% with continuous low DO levels of 0.2 mg/l over 9 moths experiment. When DO level increased to 1.0 mg/l, nitrogen removal efficiency considerably decreased. Puznava et al. (2001) reached 80% removal of total nitrogen using anoxic microfilm zones in single reactor with low DO levels of 0.5-3 mg/l. Li et al. (2008) enabled anoxic floc formation on fibrous carriers in the bottom of the reactor, achieving 63% of total nitrogen removal.

Finally, nitrification and denitrification can be performed simulateneously in a single reactor system by using aerobic denitrifiers in an aerobic environments. Aerobic denitrification has not been widely studied. Chang et al. (2011) achieved 90% removal of total nitrogen in aerobic system with heterotrophic aerobic denitrifier *Thauera mechernichensis*. However, aerobic denitrification has not been fully tested, since denitrification in many cases could occur within the anoxic zones in the flocs.

2.4. Reactor types

Bioreactors can be divided in two groups according to microbial growth. In suspended biomass reactors, bacteria grow up freely in the solution and are continuously removed with effluent. In the biofilm reactors, biomass is attached on a carrier material and thus retained in the reactor. Figure 2.4.1 shows different reactor types based on bacterial growth and hydraulic configuration. Principles, typical use, advantages and challenges of each reactor configuration are described in literature (Rittman & McCarty 2001).

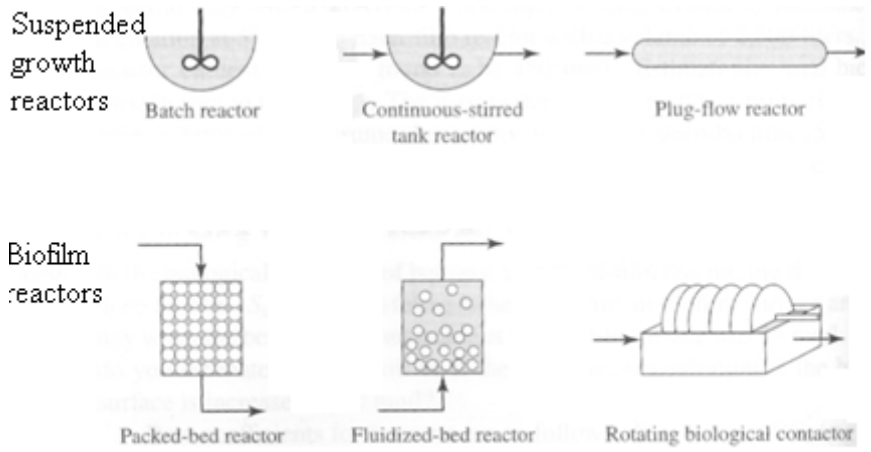

Figure 2.4.1. Different types of suspended growth and biofilm reactors, adapted from (Rittman & McCarty 2001)

2.4.1. Fluidized bed reactor

Fluidized bed reactors (FBR) are studied and compared with other biological systems more detailed by Papirio et al. (2012). FBRs are based on small carrier particles expanded inside the reactor by the continuous recirculation of the treated solution. Cells are immobilized on the carrier and thus retained in the reactor. Attachment and growth of the bacteria on the particle changes particle density, size and shape, which has to be considered in flow design (Diez Blanco et al. 1995).

Fluidized bed reactors have many advantages over other biological reactors. For more detailed comparison see Papirio et al. (2012, table 4). The use of fluidized bed reactors can lead to high biomass concentration in relatively small volumes, good substrate and biomass mixing efficiency and high substrate utilization rate (Nicolella et al. 1997). The large surface area of fine-grained carrier materials provides a lot of space for biomass immobilization. It also guarantees sufficient solid retention time (SRT) and low hydraulic retention times (HRT). FBRs can be almost ten times more efficient compared to stirred tank reactors and activated sludge systems of the same volume (Rabah & Dahab 2004).

FBRs can be operated both in up-flow and down-flow mode, according to the direction of the flow in the reactor, Fig. 2.4.1.1 (Papirio et al. 2012). Traditional FBRs are operated in up-flow mode, Fig. 2.4.1.1.a. A carrier material with higher density than water is used. Continuous flow from the bottom to the top of the reactor keeps the particles in suspension and prevents them to escape from the bottom. Down-flow FBRs, Fig. 2.4.1.1.b, are performed with the opposite flow direction and carrier particles with lower density than water are used (Sowmeyan & Swaminathan 2008).

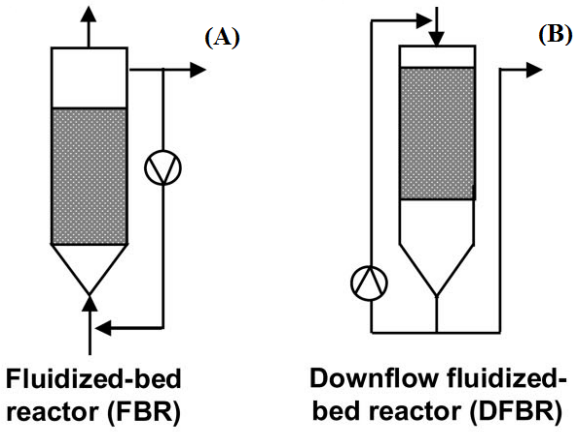

*Figure 2.4.1.1. Scheme of an up-flow FBR (a) and a down-flow FBR (b). (*Papirio et al. 2012)

2.4.1.1 Activated carbon as a carrier material

The most important features that a carrier material should satisfy are the large surface area and its durability. Papirio et al. (2012) reviewed different carrier materials used for FBR processes. Porous glass beads, granular activated carbon, silicate mineral sand, celite particles and polymeric granules covered with iron dust showed good biomass attachment during the operation of up-flow FBRs.

Activated carbon is a highly porous material with extensive surface area. Its structure results from removal of smaller molecules from carbon containing precursor material (for example wood and coal) by heating. Structure is characterized by micropores (<2 nm), mesopores (2-50 nm) and macropores (>50 nm). (Rodríguez-Reinoso 2001). Pore size is important for cell attachment. Pores have to be large enough to fit cells in, but small enough to prevent their washout (van der Meer et al. 2007).

Continuous fluidization causes friction between particles that can damage carrier material. Van der Meer et al. (2007) found better durability and biological activity results with activated carbon as a biomass carrier than with celite and Al_2O_3 in iron oxidizing fluidized bed reactors.

2.4.2. Membrane bioreactor

In this study membrane bioreactors (MBR) refer to membrane filtration bioreactors. They are suspended growth reactors where biomass is retained in the reactor and effluent filtrated through a porous membrane. Cells are freely suspended in the solution, not immobilized on the membrane. Figure 2.4.2.1 displays the most essential parts of a MBR, such as the influent inlet, the mixing system for keeping the microorganisms in suspension, the aeration system, the membrane and the effluent outlet.

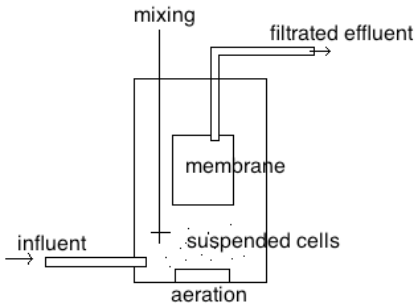

Figure 2.4.2.1. Structure of MBR.

MBR configuration has many advantages. First of all, bacteria are not washed out from the reactor and SRT can be high. Compared to batch reactors, continuous removal of smallest cell products in MBRs reduces product inhibition (Fiechter 1991). Major MBR disadvantages are the cost of membrane installation, the pumping of water through the membrane and the fouling. (Rittman & McCarty 2001).

Filtration performance of MBR decreases with time due to the attachment of particles on the membrane surface. Fouling is one of the major challenges in MBR technology. Membranes have to be cleaned and checked regularly, which increases operational costs. (Le-Clech et al. 2006). Le-Clech et al. (2006) widely reviewed fouling in the membrane bioreactors. Compounds deposited on the membrane form a resistant bed called "cake layer". Factors affecting fouling are presented in the Figure 2.4.2.1.1. These are the feed and biomass characteristics including extracellular polymeric substances (EPS), operational conditions including aeration and filtration modes and membrane characteristics including pore sizes and hydrophobicity. (Le-Clech et al. 2006)

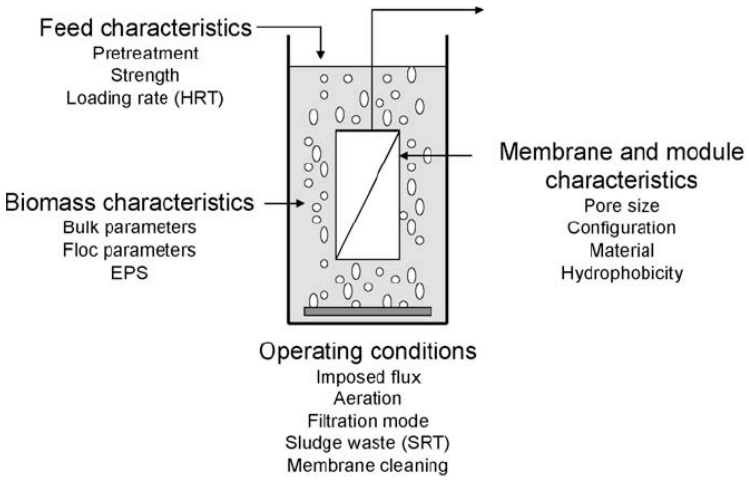

Figure 2.4.2.1.1. Factors influencing MBR fouling (Le-Clech et al. 2006)

2.4.2.1.1 Membrane characteristics

Pore size, distribution and hydrophobicity are the most important membrane characteristics for fouling. However, after formation of cake layer, the characteristics of layer become more significant (Lee & Rittmann 2002).

Pore size defines if particles enter the pores or just stay on the pore surface. Larger pores allow more particles to enter the pores, creating more persistent cake layers. On the other hand, small pores collect more particles on the layer and clogs membrane faster. (Chang et al. 2001; He et al. 2005, cited in Le-Clech et al. 2006). Narrow pore size distribution and high porosity have reduced membrane fouling in many different biological suspensions. (Marshall et al. 1993 and Matthiasson 1983, cited in Le-Clech et al. 2006).

Hydrophilic and hydrophobic characteristics of the membrane give an idea of what kind of particles attach on the membrane surface. As cells are often hydrophobic, smooth hydrophilic membranes reduce fouling. (Marshall et al. 1993 and Matthiasson 1983, cited in Le-Clech et al. 2006). If solution contains significant concentrations of hydrophilic EPS, then hydrophobic membranes are shown to foul less (Fang & Shi 2005, cited in Le-Clech et al. 2006). Membrane characteristics can be changed during the operation of the reactor by promoting the attachment of ferric hydroxide flocs, polar groups or TiO_2 nanoparticles on the membrane (Le-Clech et al. 2006). Also cell membranes can be altered, different carbon sources and pH environments change surface charges of the cell membranes and thus also fouling properties (Ohmori, Glatz 2000).

Structure of the membrane is also important. For example sponge-like membrane structures are more sensitive to fouling than smooth membranes with uniform pores (Fang & Shi 2005). Hollow fibre and flat sheet membranes have different optimal configurations, for examples different backwashing times, for minimal fouling (Cui et al. 2003, cited in Le-Clech et al. 2006).

2.4.2.1.2 Biomass and feed characteristics

Small colloids and soluble microbial products (SMP) block membranes pores. Larger suspended solids, including cells and attached EPS, form structures that inhibite dissolution of the cake layer back to the solution (Itonaga et al.2004, cited in Le-Clech et al. 2006). EPS have been reported to be the most significant factor in the fouling (Chang et al. 2002; Cho & Fane 2002, cited in Le-Clech et al. 2006).

Low temperatures often promote fouling. It causes increase in sludge viscosity and amount of EPS released to solution and decrease in biomass floc sizes. In addition, diffusion from membrane back to suspension decreases and biodegradation decelerates, resulting in higher solute concentrations. (Jiang et al. 2005, cited in Le-Clech et al. 2006).

2.4.2.1.3 Operational conditions

Cake layer formation and production of EPS can be controlled also with operational parameters. Aeartion, SRT and filtration mode are shown to significantly affect fouling mechanisms. If fouling cannot be avoided, additional cleaning prevents total membrane clogging.

Solid retention time (SRT) is an important operational parameter, since it strongly affects EPS and SMP formation. EPS and SMP concentrations decrease significantly with higher SRT in the reactor (Brookes et al. 2003; Hernandez Rojas et al. 2005, cited in Le-Clech et al. 2006). This is explained by lower organic carbon concentration in EPS and possible low biomass generation. Minimal fouling was achieved with 30 days SRT (Hernandez Rojas et al. 2005, cited in Le-Clech et al. 2006). However, high SRT can result in progressive accumulation of non-biodegradable materials in the reactor. (Le-Clech et al. 2006).

Aeration scours membrane surface and prevents large particle attachment. It also controls production of EPS (Ji & Zhou 2006, cited in Le-Clech et al. 2006). If biofilm on the membrane becomes thick enough, some inner parts of the layer can turn in anaerobic. This increases extraction of EPS and further biofilm growth and fouling on the membrane. Sufficient aeration with high DO levels can prevent anaerobic layer formation and decrease cake layer resistance. (Zhang et al. 2006, see Le-Clech et al. 2006). Multiple-hole aeration system, distributed evenly in the reactor, has shown the best aeration results (Mayer et al. 2006).

Filtration through the membrane can be controlled in two ways. Either transmembrane pressure (TMP) or flow rate are kept constant. Constant TMP keeps vacuum pressure steady. Fouling of the membrane causes decrease in flow rate. This slows downs also fouling and thus the fouling process is self-limiting. In the other method TMP is first modest, but after membrane is blocked enough, TMP increases rapidly and fouling becomes self-accelerating. Thus, with constant flux operaion, modest flow rates and frequent cleaning are needed. (Le-Clech et al. 2006).

Cleaning of the membrane is necessary when preventive actions are not enough. Chemical and physical cleaning can be used. Chemical cleaning includes the addition of compounds that remove the attached particles (Chang et al. 2002). MBR companies have their own protocols for chemical cleaning. Sodium hypochloride (NaOCl) is generally used for hydrolysis of the organic compounds and citric acid for inorganic compounds. (Le-Clech et al. 2006). Physical cleaning consists of membrane relaxation and backwashing. During membrane relaxation, filtration is paused frequently allowing attached compounds to dissolve back to the solution. In backwashing, filtrated permeate is pumped back to reactor periodically to clean the membrane. Duration, intensity and frequency of backwashing determine its effectiveness. For example backwashing of 10 minutes frequency with 45 seconds duration has shown better results than 3 minutes frequency with 15 seconds duration (Jiang et al. 2005). Backwashing increases operational costs due to the permeate loss back to reactor.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Source of microorganisms

Activated sludge (2.53 g VSS/l) from municipal wastewater treatment plant in Helsinki, Finland, was used as source of microorganisms for denitrification in fluidized bed reactors (DFBR1-3) (Papirio et al. 2013). Nitrifying FBR and MBR were seeded with activated sludge (1.84 g VSS/l) from municipal wastewater treatment plant in Tampere, Finland.

3.1.2. Nutrient stock solution

Nutrient stock solution, was prepared with mQ-water, stored in 4°C and diluted 40 times when used for preparing the feed solutions. Nutrient concentrations in the final solutions were 50 mg/l KH_2PO_4, 20 mg/l $CaCl_2 \cdot 2H_2O$, 150 mg/l $MgCl_2 \cdot 6H_2O$, 0.10 mg/l $Na_2MoO_4 \cdot 2H_2O$, 1.75mg/l $MnCl_2 \cdot 4H_2O$, 0.05 mg/l $CoCl_2 \cdot 6H_2O$.

3.1.3. Heavy metal stock solutions

Metal stock solutions for toxicity tests were prepared for Co, Ni, Cu and As in concentration of 2000 mg/L. Co, Ni and Cu were added as chloride salts, whereas As as sodium arsenite. The solutions were then acidified with 0.15 % HCl to prevent metal precipitation. Stock solutions were stored 7 °C.

3.1.4. Sodium phosphate buffer

Sodium phosphate buffer was prepared according to CSH (2006), adjusting pH to 7.0. Stock solution of 1 M and 0.25 M were prepared diluting 138 g of $Na_2HPO_4 \cdot H_2O$ and 35.5 g of Na_2HPO_4 in separate volumetric flasks to 1 and 4 litres of mQ, respectively.

3.2. Analytical methods

3.2.1. Alkalinity, pH and Dissolved Oxygen

Non-filtrated samples were used for pH and alkalinity analyses. Dissolved oxygen was measured directly from the reactors. Before each analysis pH meters were calibrated with pH 7 and pH 4 buffer solutions (FF-Chemicals, Finland). In nitrifying applications, dissolved oxygen and pH analyses were performed with a HQ 40d multi –meter

equipped with LDO101 and pHC101 electrodes (HACH, USA). In denitrification reactors and batch experiments, pH was monitored by a pH330i pH-meter (WTW, Germany) with a SenTix 41 electrode (WTW, Germany). Total alkalinity was analysed according to SFS-EN ISO 9963-1 standard potentiometric method using TITRONIC basic titration burettes (Schott, Germany). Sample was titrated with hydrochloride acid (0.102 mol/l) until pH reached 4.5.

3.2.2. Nitrate and nitrite analysis

Nitrate and nitrite were analysed with liquid chromatography (IC, Dionex Dx-120 with IonPac AS23 RCFIC column) according to SFS-EN ISO 10304-2 standard. Samples were filtered through 0.2 µm Chromafil Xtra PET-20125 filters (Macherey-Nagel, Germany) and if necessary, diluted with mQ water. Used nitrate concentrations for calibration differed from the SFS-EN ISO 10304-2 standard. They were 2, 5, 10, 20, 50, 100, 200 and 300 mg nitrate and nitrite/l.

3.2.3. Ammonium analysis

Ammonium concentration was analysed with Kjeldahl method according to SFS-EN 25663 standard. Ordior Kljeltec^{TM} 2100 (FOSS, Denmark) distillation unit and TITRONIC basic titration burettes were used.

3.2.4. Ethanol analysis

Ethanol concentration was analyzed with liquid chromatography. Samples were filtered through 0.2 um Chromafil Xtra PET-20125 filters (Macherey-Nagel, Germany) and if necessary, diluted with mQ water. A high pressure liquid chromatograph (HPLC) equipped with a RID-10A detector (Shimadzu) and a Rezer RHM-Monosachharide H^+ -column (Phenomenex) was used.

3.2.5. Metal analysis

Atomic absorption spectrophotometer AAnalyst 400 (PerkinElmer, USA) with Lumina lamps As and Co-Cr-Cu-Fe-Mn-Ni (PerkinElmer, USA) was used for analysing dissolved metals. Samples were filtered through 0.2 um Chromafil Xtra PET-20125 filters (Macherey-Nagel, Germany) and if necessary, diluted with 0.07 M nitric acid.

3.2.6. Total suspended solids and volatile suspended solids analysis

Total suspended solids and volatile suspended solids were analysed according to SFS 3008 standard. Scaltec SBC31 scale (Denver Instrument, Germany), oven (Memmert, Germany) and MR170E furnace (Heraeus Electronics, Germany) were used.

3.2.7. Microbial communities

Microorganisms were identified with 16s rRNA sequencing technique. Protocol according to Kolehmainen et al. (2007) was used for DNA extraction and polymerase chain reaction (PCR) with few modifications. Biomass samples of 30 ml, containing both solution and carrier material, were filtrated with Cyclopore track etched 0.2 um membranes (Whatman, USA) for DNA analysis. DreamTaq DNA Polymerase and Ba357F-GC primer (5'–CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCAGCAG -3') were used for PCR. Safe Imager Blue Light Transilluminator (Invitrogen), PowerShot A640 camera (Canon) and 1 % (w/v) agarose gel with Sybr® Safe DNA gel stain were used with gel electrophoresis to visualize DNA yield.

Denaturing Gradient Gel Electrophoresis (DGGE) was accomplished according to protocol of Koskinen et al. (2007) with few modifications. Denaturing gradient in polyacrylamide gel was 30% and 70%. Gels were run at 60°C and 100V for 20 hours. Forward primer Ba357F (5'–CCTACGGGAGGCAGCAG -3') was used in PCR after DGGE. Sequence data was analysed with Ridom TraceEdit software (version 1.0 for Windows, Ridom, Germany) and compared to National Center for Biotechnology Information (NCBI) GenBank database.

3.3. Reactor experiments

3.3.1. Denitrification in fluidized bed reactor

Denitrification fluidized bed reactor (DFBR) configuration is presented in the Figure 3.3.1.1.A. Synthetic feed solution, sotored in a fridge at 7°C, was pumped from influent canister to the reactors continuously with peristaltic pump (7554-77 pump with 77200-60 pump head and EasyLoadII Masterfelx L/S Model solid state speed control, Cole-Parmer Instrument Co, USA). The reactor volume was 1.1 litres and granular activated carbon (GAC) (Calgon carbon corporation, USA) was used as biomass carrier. Fluidization was maintained with continuous recirculation of the solution with another peristaltic pump (7553-77 pump with 77200-62 pump head and solid state speed controller Masterflex L/S, Cole-Parmer Instrument Co, USA). The effluent came out from the upper part of the reactor, passing through a water level adjuster and then collected in a final canister.

Reactor 1 (DFBR1) was operated at 7°C, whereas reactors DFBR2 and DFBR3 were operated at room temperature (21-22°C). DFBR1 and DFBR2 were used to study denitrification. DFBR3 was used for biomass enrichment for the batch assays.

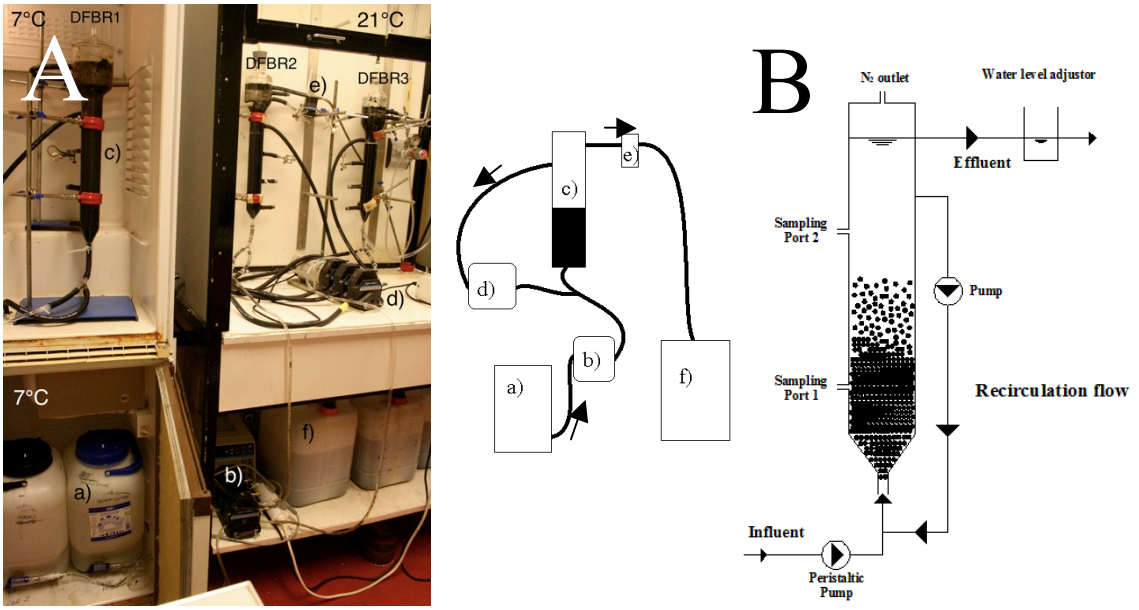

Figure 3.3.1.1.A: Configuration of DFBR: a) influent solution canister, b) influent solution pump, c) fluidized-bed reactor, d) recirculation pump, e) water level adjustor and f) effluent canister

Figure 3.3.1.1.B. Particulars of FBR carbon and e) water level adjuster (Papirio et al. 2013)

Start up –phase of DFBR reactor is described by Papirio et al. (2013). Table 3.3.1.1 shows composition and pH variations in the feed solution. Fresh solution was prepared two times per week in 20 litre tanks using tap water.

Table 3.3.1.1. Composition and pH values in denitrification influent solutions.

	Days	DFBR1 and DFBR2	DFBR3
Ethanol (99.5 % Etax Aa)	0-43 43-123 123-355	172.5 mg/l (3x stoichiometric ratio) 61.83 mg/l (1x stoichiometric ratio) 123.66 mg/l (2x stoichiometric ratio)	
NO_3^-	0-355	200 mg/l	
40x Concentrated nutrient stock solution	0-355	Chapter 3.1.2	
pH	0-43 43-179 179-197 197-211 211-225 225-242 242-256 256-295	7.5 7.0 6.0 5.5 5.0 4.5 4.0 3.5	
	295-309 309-323 323-368	3.0 2.8 2.5	4.0

During the first 43 days, influent pH was slightly alkaline (pH 7.5). Subsequently, during days 43-179, pH 7 was used and then it was gradually decreased every 2-4 weeks until 2.5 on day 323. Three different ethanol concentrations were used during the operation of DFBR1 and DFBR2. Ethanol concentrations were calculated based on equation 2.3.2.1. One mole of nitrate consumes 0.417 moles of ethanol. During the start-up period (0-43 days), ethanol concentration in influent was three times higher than the stoichiometric one (172.5 mg/L). During days 43-123, ethanol was fed in stochiometric ratio to nitrate and from day 123 onwards ethanol concentration was doubled. Samples for pH, ethanol, nitrate and nitrite analysis were taken two times a week. pH was measured immediately, other samples were filtered through 0.2 µm Chromafil Xtra PET-20125 filters and stored in 4°C for 1-2 weeks before analysis.

3.3.2. Nitrification in fluidized bed reactor (NFBR)

Configuration of nitrification fluidized bed reactor (NFBR) is shown in Figure 3.4.2.1. It was very similar to DFBRs, only an aeration reactor with an aeration pump (Aquarium lufter, Hoffman) was added to circulation.

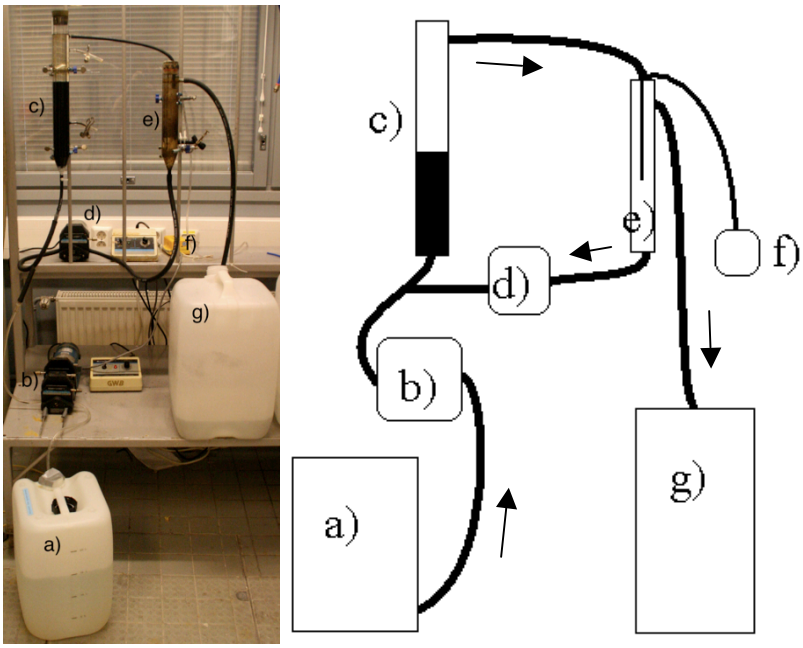

Figure 3.3.2.1 Configuration of NFBR: a) influent solution canister, b) feed solution pump, c) NFBR, d) recirculation pump, e) aeration reactor, f) aeration pump and g) effluent solution canister.

The NFBR was studied at room temperature (21°C). During the start-up phase, the system was filled with 1.4 litre of feed solution prepared according to table 3.3.2.1. Tap water was used for preparing the synthetic feed solution that was stored at 21°C. New solution was prepared 1-3 times a week in 20 litre canisters. Sodium hydrogen

carbonate was used as buffer against acidity production by nitrification (Tchobanoglous & Burton 1991).

Table 3.3.2.1. Composition of the nitrification feed solution.

Compound	Concentration (mg/l)	Corresponds to
NH_4	100	
$NaHCO_3$	1000	600 mg/l $CaCO_3$
40x concentrated nutrient stock solution	Chapter 3.1.2	

At the beginning the reactor was seeded with an activated sludge of 100 ml (chapter 3.1.1), previously stored at 4°C. Recirculation was adjusted in order to get a 25 % of fluidization. During the first 15 days, the reactor was operated in batch mode in order to enable sufficient enrichment of slowly growing nitrifying microorganisms. Feed solution was circulated between aeration and fluidized bed reactors. After 15 days, the NFBR was operated continuously with a 12-hour hydraulic retention time (HRT) and 1.94 ml/min flow rate. Samples for ammonium, pH, alkalinity, nitrate and nitrite analysis were taken 2 times a week. Ammonium, alkalinity, pH and DO were measured immediately. Samples for nitrite and nitrate analysis were filtered through 0.2 um Chromafil Xtra PET-20125 filters and stored in 4°C for 1-2 weeks before analysis.

3.3.3. Nitrification membrane bioreactor (NMBR)

Figure 3.3.3.2 presents NMBR configurations with two different mixing systems. Figure 3.3.3.2.A present mixing with continuous circulation and figure 3.3.3.2.B with continuous stirring.

Synthetic influent solution was pumped to the reactor by a peristaltic pump (7552-87 pump with 77201-60 pump head and easyLoad II Masterfelx L/S solid state speed controller). Water level controller connected with controller panel (AC771, Ordel, Turkey) maintained the water level in the reactor approximately between 5±0.05 litres. Oxygen was supplied with an aquarium aeration pump (F-2657, Europet, Germany). Effluent was filtrated either through a hollow fibre (pore size 0.2 μm, Zena, Czech Republic) or flast sheet (pore size 0.04 μm, UP 150, Microdyn-Nadir, Germany) membranes, which were attached to aeration pipe to prevent membrane clogging. Used flat sheet membrane material was Permanent hydrophilic Polyethersulfon (PES). Peristaltic pump (Sci 323, Watson-Marlow) was used for filtration and backwashing the effluent. Backwashing of frequency 10 minutes and duration of 50 seconds was controlled with same control panel used for maintaining the water level. Manometer (EN 837-1) was attached between membrane and filtration pump to monitor pressure changes.

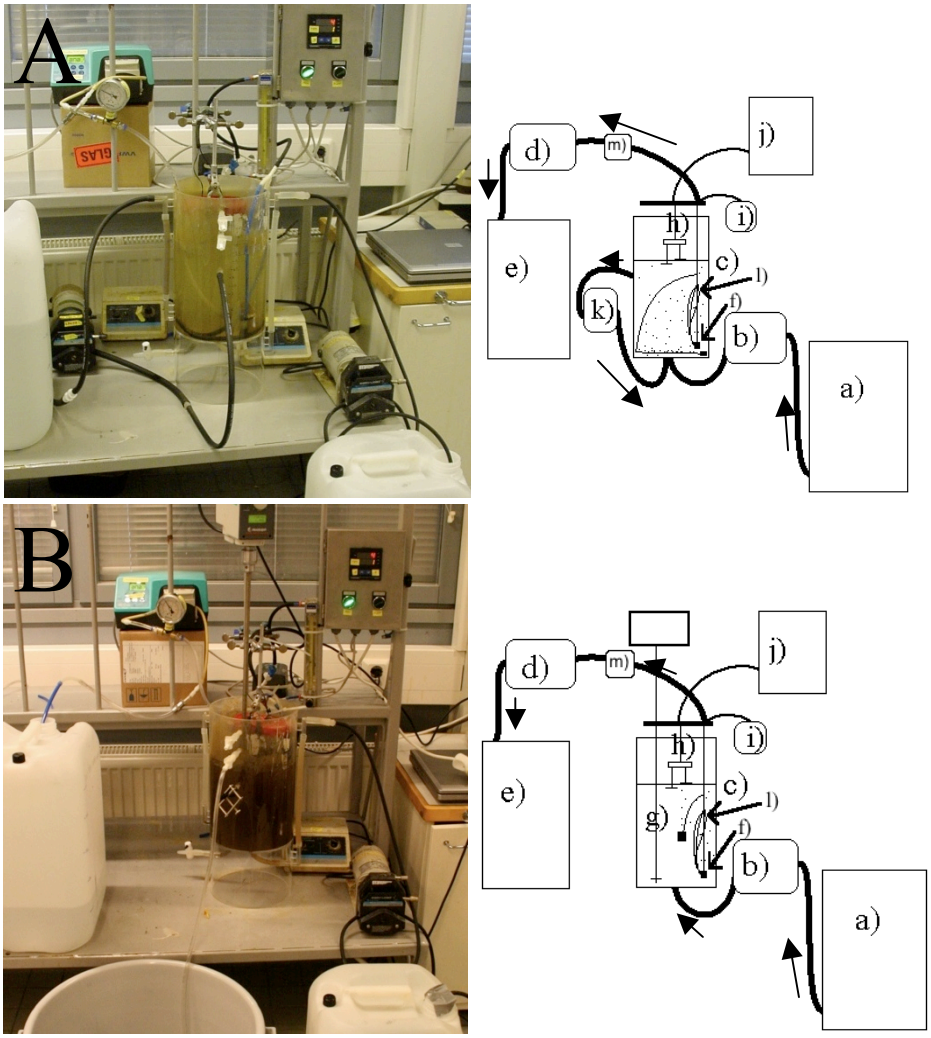

Figure 3.3.3.2. NMBR A) mixing with continuous circulation and B) mixing with a stirrer. Configuration: a) influent solution canister, b) influent solution pump, c) 5 litres reactor, d) peristaltic pump for filtration, e) effluent canister, f) two aquarium aeration pipes, g) stirrer for mixing, h) water level controller, i) aquarium aeration pump, j) controller panel for backwashing and water level, k) pumping system for continuous circulation mixing, l) hollow fibre membrane attached to an aeration pipe, m) filtration manometer and n) flat sheet membrane.

3.3.3.1 Start-up and feed

In the start-up phase, 5.4 litres of feed solution described in table 3.3.2.1 was fed to the system that was operated in batch mode for 2-3 days. 500 ml of activated sludge (Chapter 3.1.1) was taken from wastewater treatment plant and stored in 4°C for 40 days before addition to the reactor.

After start-up, first 4-7 days feed solution was continuously pumped to NMBR with a 22.5 h HRT. During days 7-17 water level adjustor was broken and reactor stayed in batch mode. During days 17-66 influent solution was added continuously with 20 h HRT. Samples were taken and analysed in similar way to NFBR experiment.

3.3.3.2 Comparison of mixing systems

Two different mixing systems inside MBR were studied: mixing with recirculation and stirring. Continuous recirculation was tested during the first 33 days. Solution was mixed with continuous circulation from the side of the reactor to the bottom. In addition to the recirculation, an aeration pipe with small holes on the sides was placed in the bottom of the reactor. This method showed a poor sludge mixing and it was changed to continuous stirring with 120 rpm on day 33.

3.3.3.3 Washing procedure for clogged membranes

In the beginning, the flat sheet membrane was kept with its support in a HCl solution (pH 2) for 15 minutes. The membrane was then rinsed with distilled water and used as a filter for distilled water for 5 minutes to remove remaining acidity. Subsequenly, membrane was detached from the support and kept in NaOH solution (pH 11) for 15 minutes. Then membrane was attached to the support again and the remaining alkalinity was removed by sucking distilled water through the membrane for 10 minutes. Membrane was stored in distilled water before it was used again in the reactor.

Washing method for hollow fibre membrane started with rinsing membrane with tap water and distilled water. Then membrane was kept in HCl (2%) for 2 hours, rinsed with distilled water, kept in NaOH (1 N) for 24 hours, rinsed with distilled water and kept in 0.4 % NaOCl for 2 hours. Finally, the membrane was again rinsed with distilled water and stored in distilled water before using it in the reactor.

3.3.4. Simultaneous denitrification and nitrification

Denitrifying and nitrifying microbes were first enriched simultaneously in separate membrane bioreactors for 2 and 14 days, respectively. Same original activated sludge (see Chapter 3.1.1) was used for both enrichments. Nitrifying microorganisms were enriched in similar reactor to NMBR. In denitrification MBR (DMBR) aeration was not used. Table 3.3.4.1 presents feed solution used for denitrification enrichment and combined denitrification and nitrification process.

Table 3.3.4.1. Feed solutions used for denitrification enrichment and combined denitrification and nitrification process.

Compound	Reactor	Concentration (mg/l)
$NaHCO_3$	DMBR	156.7
	DNMBR	783
$NaNO_3$	Both reactors	546
Ethanol	Both reactors	230.4
Nutrients	Both reactors	See Chapter 3.1.2
NH_4Cl	DNMBR	294.4

In the beginning of the experiment, 2 litres of denitrification enrichment solution and 4 litres of nitrification enrichment solution were combined in single reactor. Water level

was adjusted in order to fill 5.5 litres of the reactor. The feed solution (table 3.3.4.1) was continuously fed at a 20 h HRT.

3.3.4.1 Comparison of aeration intervals

Oxygen gradient over time was created to enable anoxic and aerated conditions for denitrification and nitrification processes, respectively. Three different aeration/non-aeration time programs were studied. First three days aeration was turned on for 60 minutes and then turned off for 20 minutes (phase I). During days 3-4, aeration was turned on for 60 minutes and off for 30 minutes (phase II). During days 5-19, aeration was turned on for 60 minutes and off for 60 minutes. Samples were taken and analysed in similar way to NFBR experiment.

3.3.4.2 Comparison of membrane types

Different flat sheet membranes, 150 kDa and 100 kDa ultrafiltration (UF) membranes and 0.2 µm microfiltration (MF) membranes (Microdyn Nadir, Germany) were used. When pressure in vacuum pump or the effluent flow rate decreased to -0.8 bar or 4.0 ml/min, respectively, membranes were washed and replaced by a similar clean membrane. Hollow fibre membrane was replaced by flat sheet membrane on day 6. Mixing with stirrer and circulation were used with flat sheet and hollow fiber membranes, respectively.

3.4. Denitrification batch bottle experiments

Effects of pH (3-7) and toxic heavy metals on denitrification were studied in batch assays. General experiment protocol is described in figure 3.3.1. Experiments are described more detailed in chapter 3.4.1 and 3.4.2.

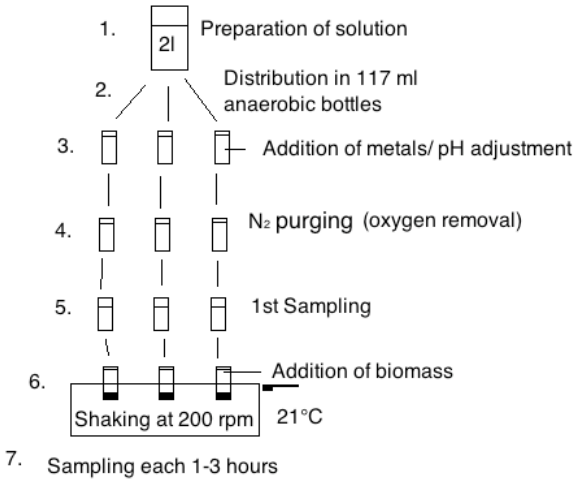

Figure 3.4.1 Batch bottle experiment protocol.

3.4.1. Effects of low pH

Solution of 110 ml, prepared according to table 3.4.1.1 was used in 117 ml serum bottles. The solution was prepared with tap water. Hydrochloric acid was used to adjust pH. Different initial pH values from pH 3 to pH 7 were used. Solutions were purged with nitrogen gas for 5 minutes to remove dissolved oxygen. Biomass was taken from DFBR3. Samples for nitrate, nitrite and ethanol concentration were taken every hour or every 1.5 hours for duration of 9 hours. Samples of 1.5 ml were filtrated through Chromafil Xtra PET-20125 0.45 um filters (Macherey-Nagel, Germany) and stored in 2 ml tubes at 4°C.

Higher pH values (pH 5.5-7) were studied first. Bottles of these experiments, including biomass and remaining solution, were stored in 4°C and reused in later experiments by replacing solution with new influent, but saving the biomass.

Table 3.4.1.1. Solution used in low pH batch assays for denitrification.

NO_3^-	200 mg/l
Ethanol	61.86 mg/l (stoichiometric ratio)
40x Nutrient stock solution	Chapter 3.1.2

3.4.2. Toxicity of arsenic

Arsenic metal toxicity was studied in a similar way to pH assays. Only different concentrations of sodium arsenite were added to replicate serum bottles. These concentration correspond to 10, 20, 50 and 75 mg As(III)/l. After addition of arsenic, the bottles were placed at 4°C for 24 hours in order to achieve complete metal precipitation. Subsequently, the pH was adjusted again to the desired values and ethanol was added. Oxygen was removed by purging solution with nitrogen gas for 15 minutes and finally 5 ml of biomass were added to bottles. Biomass was taken from DFBR3. Samples of liquid phase (5 ml) were taken before addition of biomass and after 15 min, 3h, 6h and 9h. Samples were filtrated immediately after sampling and stored at 4°C. pH was measured immediately after sampling, whereas ethanol, nitrate, nitrite and metals within one week after the end of the experiment.

3.4.3. Study of proper conditions for denitrification in batch bottles

In order to find the optimal conditions for carrying out denitrification in batch tests, preliminary tests were performed to investigate the effects of pH, buffer concentration, quantity of biomass and concentration of ethanol. Biomass was taken from DFBR3. Four different concentrations of sodium phosphate buffers (1, 10, 50 and 100 mM) were studied first. Effect of double ethanol and double biomass concentrations were studied then using 20 mM sodium phosphate buffer.

3.4.4. Toxicity of nickel, copper and cobalt

Metal toxicity batch bottle experiments for denitrification with copper (Cu^{2+}), cobalt (Co^{2+}) and nickel (Ni^{2+}) were done with solution presented in table 3.4.4.1. The solution was prepared with tap water. Ethanol, biomass and buffer concentrations were chosen according to preliminary test results (Chapter 4.2.2). Two times higher biomass concentration (10 ml of biomass in 117 ml serum bottles) was used compared to arsenic toxicity assay (Chapter 3.4.2). New biomass was taken for each of tests from DFBR3. Nickel concentrations of 1, 2, 5, 10, 50 and 100 mg/l were studied. Studied cobalt concentrations were 0.5, 5, 20, 50 and 100 mg/l and studied copper concentrations 50, 500 and 1000 mg/l.

Table 3.4.4.1. Parameters used to study metal (Ni^{2+}, Cu^{2+} and Co^{2+}) toxicities in batch bottles.

Compound	Concentration
NO_3	200 mg/l
Ethanol	123.66 mg/l (double stoichiometric ratio)
40 x Nutrient stock solution	Chapter 3.1.2
Sodium phosphate buffer (pH 7)	50 mM

4. RESULTS

4.1. Reactor experiments

4.1.1. Denitrification in fluidized bed reactors

The start-up of the denitrification reactors was studied at temperatures of 7°C (FBR1) and 20°C (FBR3). The start-up period was followed by stepwise decease of feed pH from 7-2.8. Figure 4.1.1.1 shows nitrate, nitrite, ethanol and pH evolution in reactors DFBR1, DFBR2 and DFBR3.

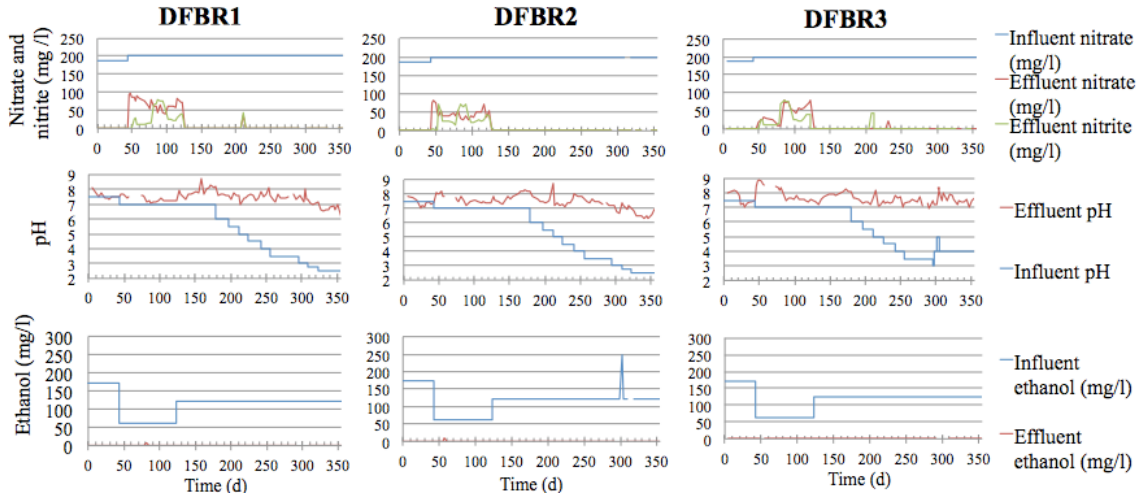

Figure 4.1.1.1. Nitrate, nitrite and ethanol concentrations (mg/l) and pH evolution in DFBR1 and DFBR2.

Nitrate and nitrite were reduced to nitrogen gas during all the experimental periods at nitrate feed rate of 870 mg/l/d and HRT of 4.5 h, except during days 46-123 when stoichiometric ethanol was supplied. The result show that pH was maintained between 6.2-8.8, 6.3-8.8 in DFBR1 and DFBR2, respectively.

4.1.2. Comparison of FBR and MBR for nitrification

Both nitrification reactors, NFBR and NMBR, were studied in room temperature. Same ammonium, nutrient and sodium phosphate buffer containing feed solution and same source of biomass were used in both processes.

Figure 4.1.2.1 presents nitrification results in NFBR and NMBR reactors. Figure 4.1.2.1 shows that nitrate production varied significantly in both reactors, even though

almost all ammonium was consumed. Averagely, pH was 7.5 in NMBR and 7.41 in NFBR, since $NaHCO_3$ buffer was used.

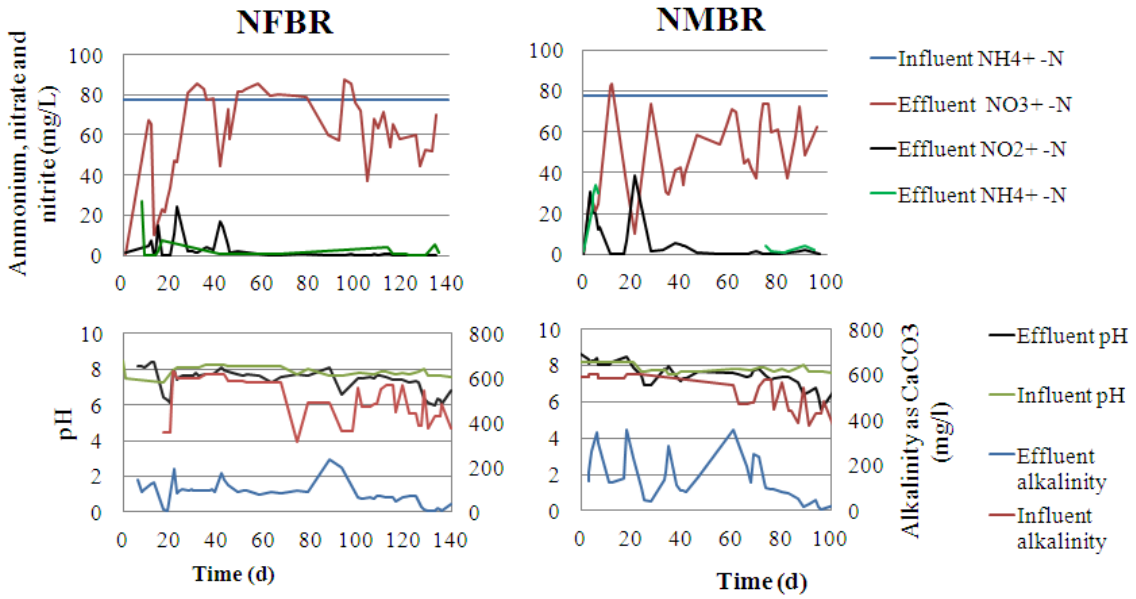

Figure 4.1.2.1. Ammonium (NH_4^+-N), nitrate (NO_3^--N) and nitrite (NO_2^--N) nitrogen, alkalinity as $CaCO_3$ (mg/l) and pH evolution in NFBR and NMBR.

Ammonium concentration was not followed regularly in the beginning, but after 75 days results showed 93.2-100 % ammonium removal in NFBR and 94.6-99.3 % removal in NMBR. Alkalinity was decreased in the reactor approximately of 400-600 mg $CaCO_3$/l.

In NMBR, cell suspension varied in the beginning of the experiment due to mixing and HRT changes. First 33 days, using continuous circulation as mixing system, significant amount of sludge settled to the bottom of the reactor. In addition, NMBR worked in batch mode during days 7-17 because of water level adjuster maitenance.

4.1.3. Simultaneous denitrification and nitrification

Simultaneus nitrification and denitrification membrane bioreactor (DNMBR) was studied in MBR reactor. Nitrifying and denitrifying microorganism were first enriched in separate MBR reactors and then combined together. DNMBR feed solution contained 100 mg/l of ammonium and was added to reactor with 20h HRT.

Figure 4.1.3.1 shows nitrification, denitrification and total nitrogen removal evolution (%) of DNMBR. Different aeration and non-aeration intervals and membrane types were compared.

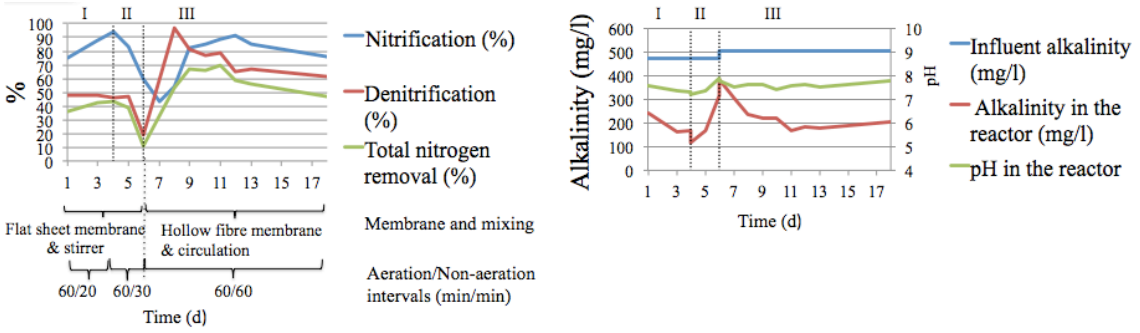

Figure 4.1.3.1. Nitrification (%), denitrification (%), total nitrogen removal (%), influent and effluent alkalinity as $CaCO_3$ (mg/l) and pH results in DNMBR reactor, with corresponding membrane type and mixing system.

As presented in the Figure 4.1.3.1, in phases I-II nitrification was 60-90 % and denitrification 46-51 %. In phase I, aeration was turned off for 20 minutes between two 60 minutes intervals of aeration. In phase II the anoxic interval was 30 minutes. This caused decrease in nitrification performance, from 95 to 60 % (days 4-6). Between day 5 and 6, denitrification quickly decreased from 50 to 19 %, as a consequence of too high DO in non-aeration period. During phase III, aeration interval was changed again. Aeration and non-aeration intervals were both 60 minutes long. Denitrification performance significantly increased from 19% to 97 % in two days. Nitrification continued to decrease from 60 to 43% (days 6-7), but recovered to 91 % on day 12. DNMBR experiment was ended after 19 days due to the low average nitrogen removal of 47%. However, maximum total nitrogen removal of 67% was achieved on day 9.

4.2. Denitrification in batch bottle assays

4.2.1. Denitrification at pH 4.8 and 5.0

Figure 4.2.1.1 shows the results obtained from batch tests. With feed pH higher than 3.5, the alkalinity produced by denitrification neutralized the pH of the solution. In 9 hours nitrate removal reached 88.1 %. With initial pH 3, denitrification partially occurred during the first three hours, increasing the pH to 4.8. Then the process was inhibited. Re-addition of stoichiometric ethanol after 4.5 hours in the bottle with initial pH of 4 resulted in a complete denitrification at the end of the experiment.

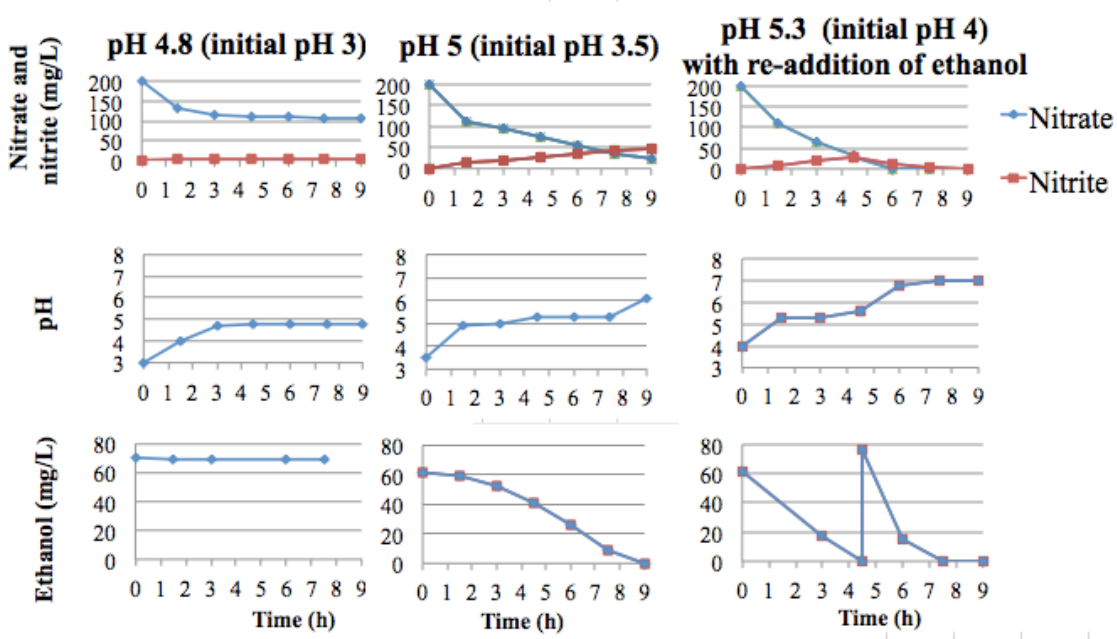

Figure 4.2.1.1. Denitrification batch bottle experiment results with pH 4.8 (initial pH 3), pH 5 (initial pH 3.5) and pH 5.3 with re-addition of ethanol at 4.5h (initial pH 4): pH evolution and nitrate, nitrite and ethanol concentrations (mg/l).

4.2.2. Optimal conditions for denitrification batch assays

In order to find the optimal conditions for performing the batch assays aimed at metal toxicity evaluation, different buffer concentrations, biomass amounts and ethanol concentration were tested. Nitrate removal percentages and pH values after 5 hours are shown in the table 4.2.2.1.

Table 4.2.2.1. pH and nitrate removal results from buffer concentration assay and biomass and ethanol concentration assays.

	pH		Nitrate removal at 5 h (%)
Buffer concentration assay	7.0-8.4	1 mM buffer	78
	7.0-7.3	10 mM buffer	72
	6.9-7.0	50 mM buffer	71
	6.8-6.9	100 mM buffer	75
Biomass and ethanol concentration assay (with 20 mM buffer)	7.0-7.2	Normal (5ml biomass /110 ml bottle, stoichiometric ratio of ethanol and nitrate, no reducing agent)	37
	7.0-7.3	123.66 mg/l (double stoichiometric ratio)	51
	7.0-7.3	Double biomass concentration (10 ml /117 ml bottle)	100
	7.0-7.4	Double ethanol and biomass	100

50 mM of buffer solution, 10 mL of biomass and 23 mg/l of ethanol resulted in the highest nitrate removal efficiencies.

4.2.3. Ni^{2+}, Co^{2+}, Cu^{2+} and As(III) toxicities for denitrification

4.2.3.1 Nickel

The effect of nickel concentration on dissolved nickel, nitrate, ethanol and nitrite concentrations in batch assay are shown in Figure 4.2.3.1.1. Soluble nickel concentration remained stable for the first hour. Then it significantly decreased between 1-3 hours.

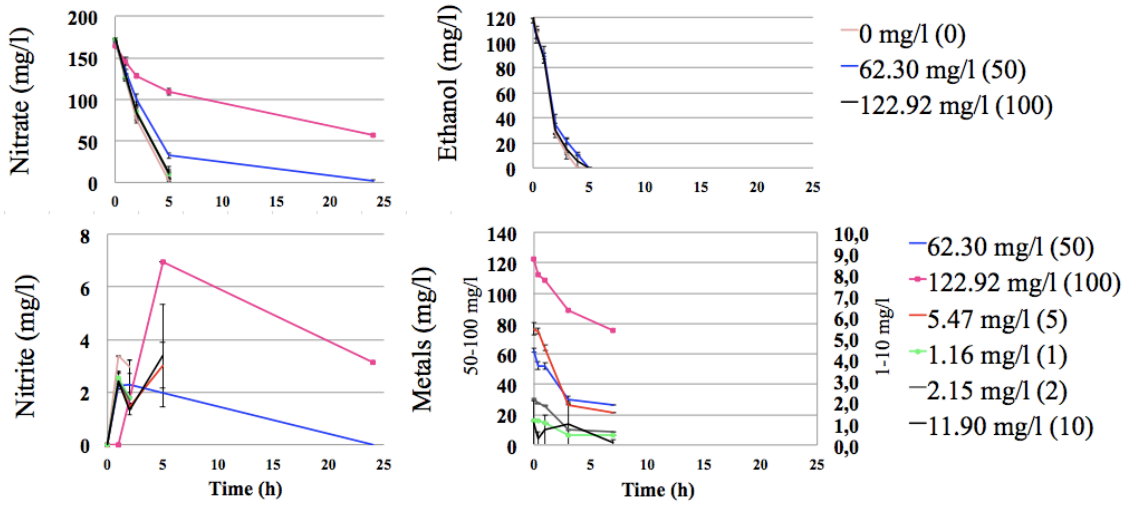

Figure 4.2.3.1.1. Nickel toxicity results from batch bottle experiments: nitrate, nitrite, ethanol and soluble metal concentrations (mg/l) with corresponding initial soluble nickel concentration, added concentrations in parenthesis.

Ethanol was consumed in the bottles in five hours. Nitrite accumulated until five hours but then it decreased in the following hours. Nitrate was almost completely removed after five hours, except at the highest initial Ni concentrations of 62.3 and 122 mg/l. After 24 hours, nitrate was removed in all the bottles besides the one with the highest initial Ni concentration of 122 mg/l, where nitrate removal only reached 65 %. Figure 4.2.3.1.2 summarises nitrate removal percentages of different initial nickel concentrations after 5 and 24 hours.

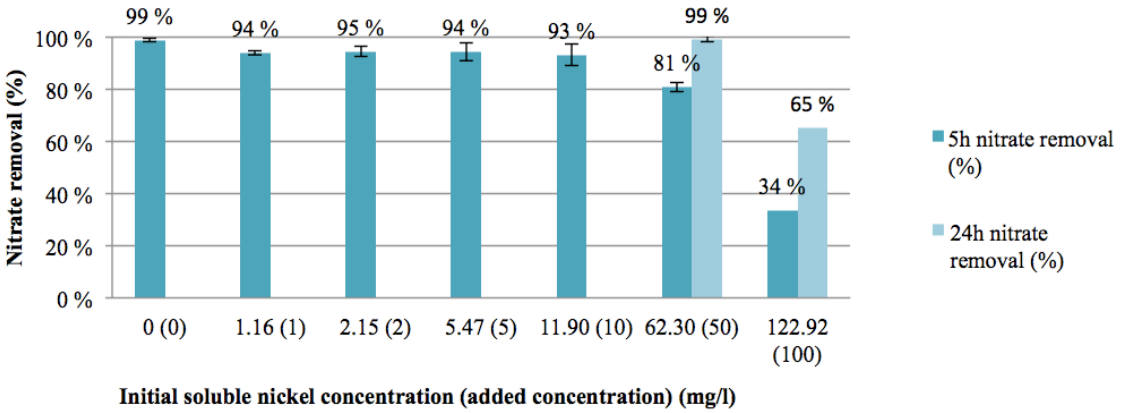

Figure 4.2.3.1.2. Nitrate removal in the nickel toxicity batch assay at 5 and 24 hours. Error bars present standard deviations of replicate experiments. Effect of the highest Ni concentration of 122.92 mg/l was tested without replicates.

4.2.3.2 Cobalt

The effect of cobalt concentration on denitrification was studied. Soluble cobalt concentration between 0.52 and 86.60 mg/l did not inhibit denitrification in 24 hours. Dissolved cobalt concentration, nitrate, ethanol and nitrite evolutions in the cobalt toxicity batch test are shown in figure 4.2.3.2.1.

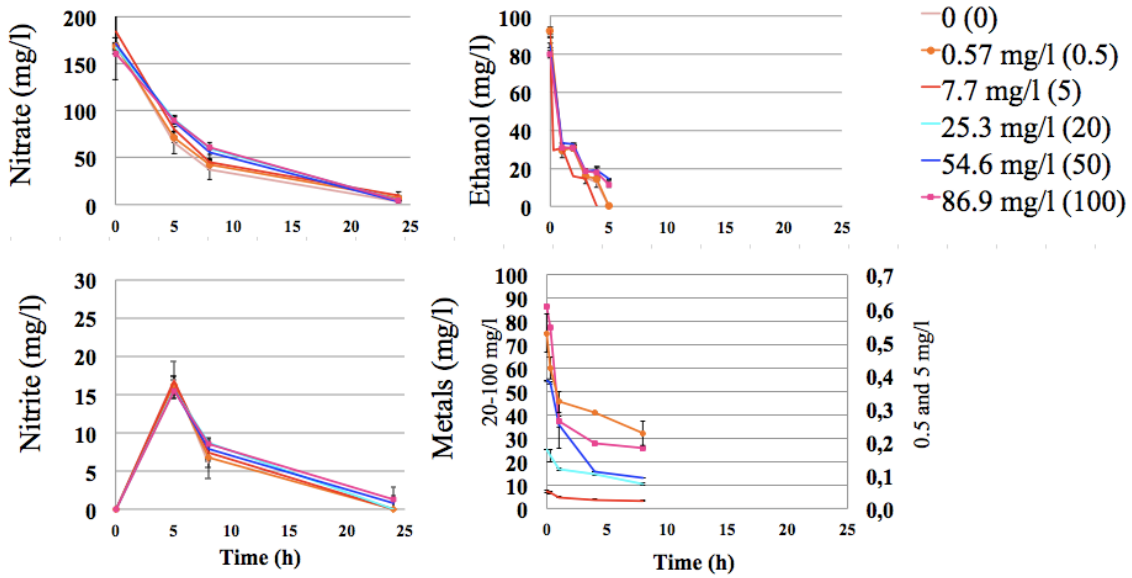

Figure 4.2.3.2.1. Cobalt toxicity results from batch bottle experiments: nitrate, nitrite, ethanol and soluble metal concentrations (mg/l) with corresponding initial soluble cobalt concentration, added concentrations in parenthesis.

Soluble cobalt concentrations decreased significantly during the first hour from the initial values of 0.52, 7.30, 25.18, 54.75 and 86.60 to 0.32, 4.92, 16.70, 36.05 and 37.15 mg/l, respectively. Cobalt concentration decreased more slowly during the rest of the experiment and, after 8 hours, it was 0.23, 3.23, 10.49, 13.08 and 25.90 mg/l, respectively, in the five bottles. Corresponding ethanol removal percentages were 100, 88.5, 86.5, 82.9 and 85.5 % at 8 hours. After 24 hours, nitrate was removed from all the bottles. Nitrite accumulated in solution until 5 hours and then nitrite concentration quickly decreased. Figure 4.2.3.2.2 summarises nitrate removal percentages with different initial cobalt concentrations at 5 and 24 hours.

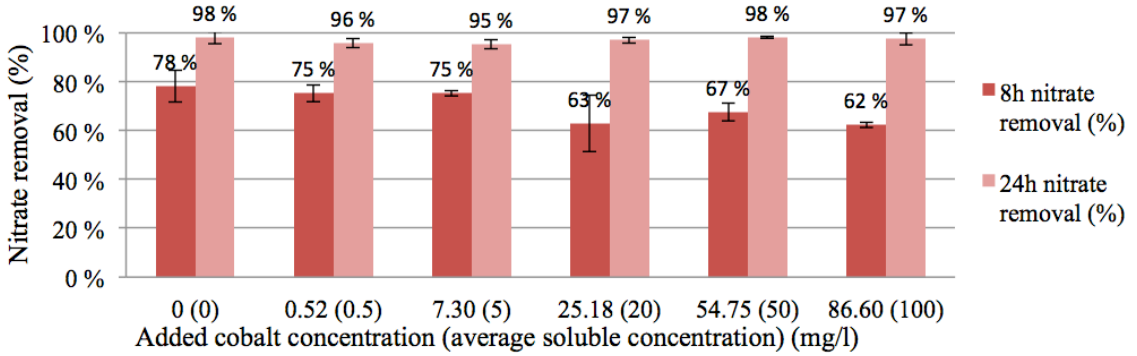

Figure 4.2.3.2.2. Nitrate removal in the cobalt toxicity batch assay at 5 and 24 hours. Error bars present standard deviations of replicate experiments.

4.2.3.3 Copper

The effect of copper concentration on denitrification was studied. Figure 4.2.3.3.1 shows the profiles of nitrate, ethanol, nitrite and copper during the copper toxicity batch test. Soluble copper concentrations were below 1 mg/l during all the experiment. Added concentration of 50, 500 and 1000 mg/l decreased to 0.77, 0.23 and 0.35 mg/L, respectively, before the addition of biomass. Copper concentration below 0.5 mg/l were not reliable due to the detection limit of the AAS analysing method.

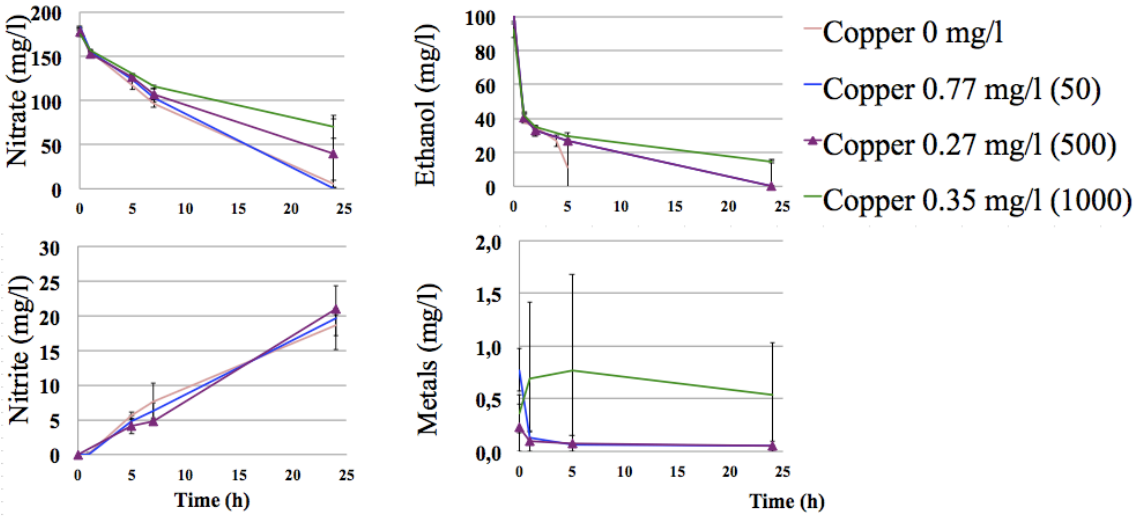

Figure 4.2.3.3.1. Copper toxicity results from batch bottle experiments: nitrate, nitrite, ethanol and soluble metal concentrations (mg/l) with corresponding initial soluble copper concentration, added concentrations in parenthesis.

During the first hour, ethanol removal percentages were 62.9, 60.6, 59.0 and 54.4 % in the bottles containing 0, 50, 500 and 1000 mg/l of copper, respectively. Then ethanol consumption rate considerably decreased. After 24 hours ethanol was consumed in the solutions containing 0 and 50 mg/l of initial copper, whereas ethanol removal was 88.1% and 84% in the bottles with initial Cu concentration of 500 and 1000 mg/l,

respectively. Nitrite concentrations constantly increased in all the bottles, reaching the highest values of 18.6, 19.6 and 21.01 mg/l in solutions containing initial Cu of 0, 50, 500 mg/l, respectively, after 24 hours.

Figure 4.2.3.3.2 summarises nitrate removal in copper toxicity assay. At 7 hours nitrate removal was 47, 44, 40 and 34 % in bottles containing 0, 50, 500 and 1000 mg/l of precipitated copper. Removal was 97, 100, 77 and 60 % at 24 hours, respectively.

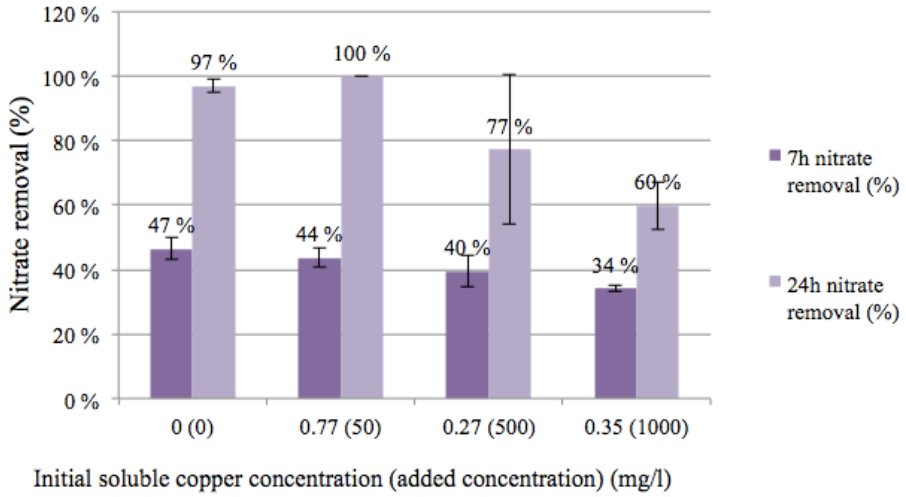

Figure 4.2.3.3.2. Nitrate removal (%) in the copper toxicity batch assay at 7 and 24 hours. Error bars present standard deviations of replicate experiments.

4.2.3.4 Arsenic

The effect of arsenic concentration on denitrification was studied. Dissolved arsenic concentration, nitrate and ethanol consumption and nitrate formation in the arsenic toxicity batch assay are presented in the figure 4.2.3.4.1.

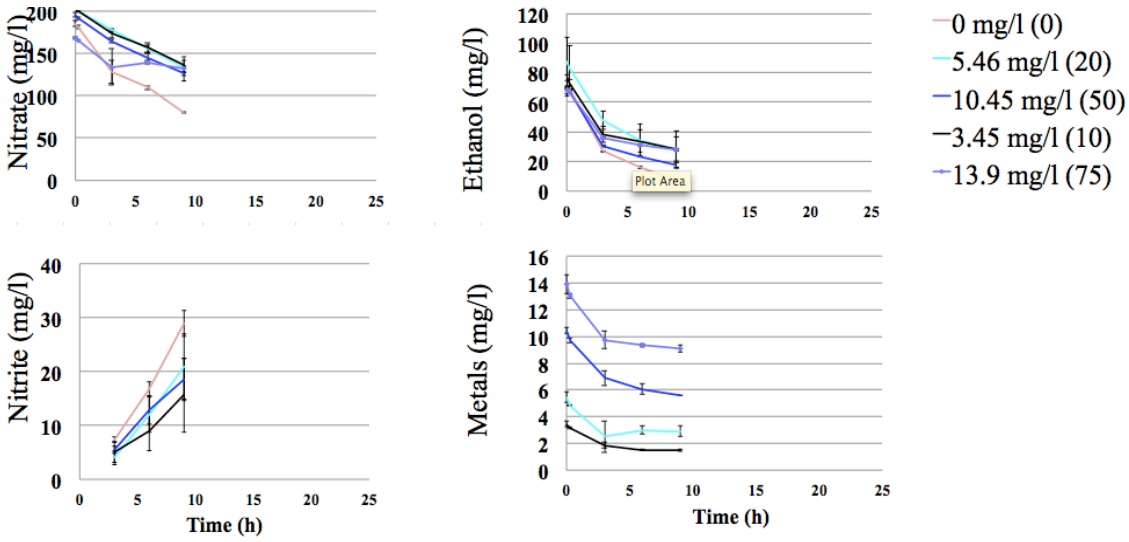

Figure 4.2.3.4.1. Arsenic toxicity results from batch bottle experiments: nitrate, nitrite, ethanol and soluble metal concentrations (mg/l) with corresponding initial soluble arsenic concentration, added concentrations in parenthesis.

Figure 4.2.2.4.2 presents pH evolution during the arsenic experiment. No buffer was used and thus pH increased in the solution over time

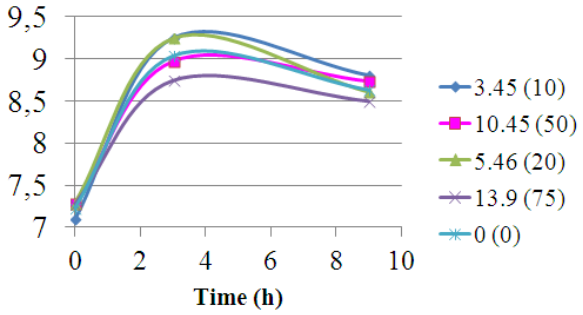

Figure 4.2.3.4.2. pH values in arsenic metal toxicity test according to initial soluble total arsenic.The added As(III)concentrations are shown in the parenthesis

Samples were taken only during the first 9 hours. After three hours, ethanol removal was 59.6, 49.7, 45, 56.1 and 47.5 % in bottles containing initially 0, 3.45, 5.46, 10.45 and 13.9 mg As/l, respectively. After nine hours, ethanol removal percentage increased to 88.7, 63, 67.9, 74.8 and 59.5 % respectively. Nitrite concentrations were the highest after nine hours.

Figure 4.2.3.4.3 summarises nitrate removal in arsenic toxicity assay. Nitrate was 57, 33, 35, 35 and 22 % removed in bottles containing initially 0, 3.45, 5.46, 10.45 and 13.9 mg As /l, respectively.

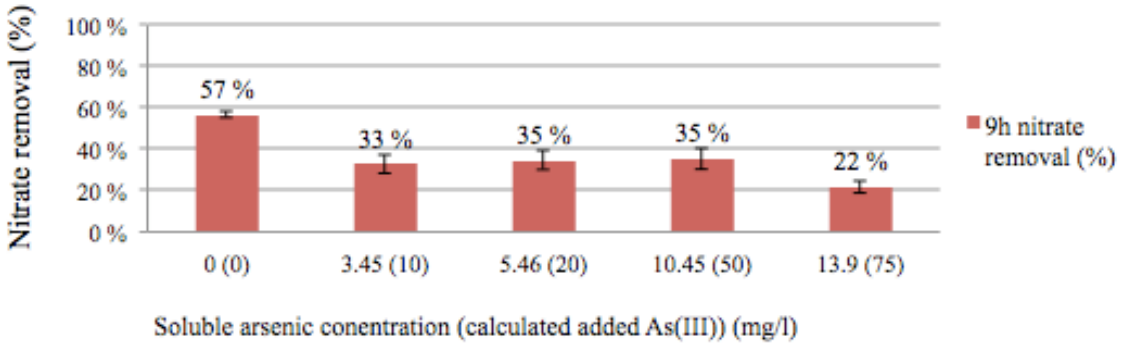

Figure 4.2.3.4.3. Nitrate removal (%) in the arsenic toxicity batch assay at nine hours. Error bars present standard deviations of replicate experiments.

4.2.3.5 Summary of metal toxicities

Table 4.2.3.5.1 summarises nitrate removal rates for each of the metal toxicity assay. Metal concentrations are presented as soluble concentrations, added concentrations are in parenthesis. Linear regressions (trendlines) for nitrate evolution, presented in figures 4.2.3.1.1, 4.2.3.2.1, 4.2.3.3.1 and 4.2.3.4.1, were calculated with Microsoft Excel for Mac 2011 (Version 14.2.3). Nitrare removal was calculated from linear regression coefficient, taking into account solution volumes. Thus rates are expressed as mg NO3-/h/ 5 or 10 ml of biomass. Coefficients of determination (R2) of linear regression are mentioned after each calculated removal rate.

Table 4.2.3.5.1. Removal rates (V_{As} = mg NO_3/ h/ 5 ml biomass, $V_{Ni,} = V_{Co} = V_{Cu}$ = mg NO_3/ h/ 10 ml biomass) and their coefficients of determination (R^2) for each metal toxicity assay. Added concentrations are presented in the parenthesis.

As (mg/l)	Vas	R^2	Ni (mg/l)	VNi	R^2	Co (mg/l)	Vco	R^2	Cu (mg/l)	Vcu	R^2
13.9 (75)	0.43	0.72	122.92 (100)	0.34	0.98	86.9 (100)	1.39	0.99	0.35 (1000)	0.74	1
10.45 (50)	0.85	0.99	62.30 (50)	2.95	0.99	54.6 (50)	1.60	0.99	0.27 (500)	0.81	0.99
5.46 (20)	0.85	1	11.90 (10)	3.47	0.97	25.3 (20)	1.49	0.99	0.77 (50)	0.96	1
3.45 (10)	0.80	0.99	5.47 (5)	3.49	0.98	7.7 (5)	1.94	0.98			
			2.15 (2)	3.48	0.97	0.57 (0.5)	1.78	0.98			
			1.16 (1)	3.46	0.97						
0 (0)	1.28	0.96	0 (0)	3.61	0.97	0 (0)	1.88	0.97	0 (0)	1.08	0.99

Figure 4.2.3.5.1 presents decrease of denitrification rates (%) in metal toxicity batch assays in relation to added and soluble metal concentrations. Soluble copper and arsenic concentrations were significantly lower than added concentrations.

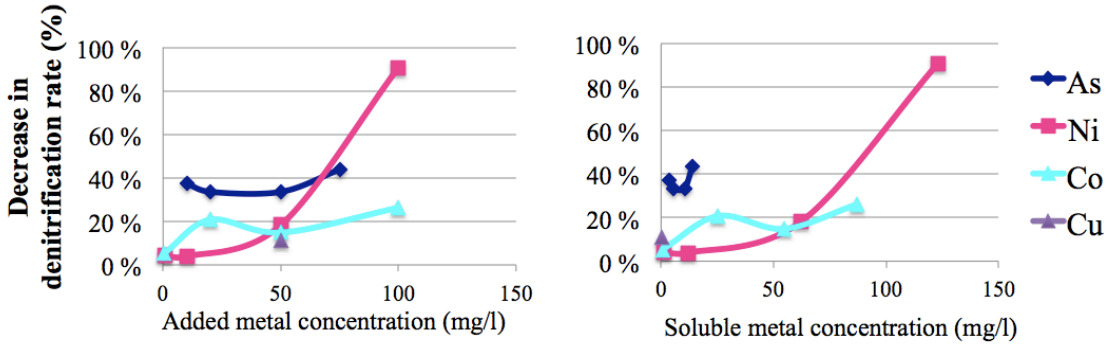

Figure 4.2.3.5.1 Decrease in denitrification rates (%) in copper, cobalt, nickel and arsenic toxicity assays.

Figure 4.2.3.5.2 presents summary of metal toxicities with addition of 50 and 100 mg/l of metals. Arsenic and copper were not studied with 100 mg/l concentrations.

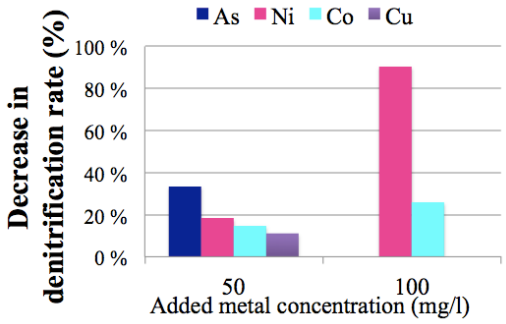

Figure 4.2.3.5.2 decrease in denitrification rate with addition of 50 mg/l of arsenic, nickel, cobalt and copper and 100 mg/l of nickel and cobalt.

In summary, the toxicity of metals towards denitrification increases in the following order Cu < Co <Ni <As.

4.3. Microbial communities

Figure 4.3.1 presents partial 16s rRNA DGGE bands from reactors DFBR1-3, NMBR, NFBR, the original activated sludge used for nitrification and nitrification enrichment for DNMBR. Microorganisms from denitrification reactors were purified twice, at days 279-281 (influent pH 3.5) and 342-343 (influent pH 2.5) of reactor operation. Samples from NFBR and NMBR were obtained at days 35 and 34, respectively. Two replicate PCR products of each sample were studied. Bands with succeful sequencing results are marked with numbers 41-116. Sequencing of some bands did not succeed, thus some bands were estimated based on bands in other samples, existing in same levels in figure 4.3.1. These estimations are presented in parenthesis.

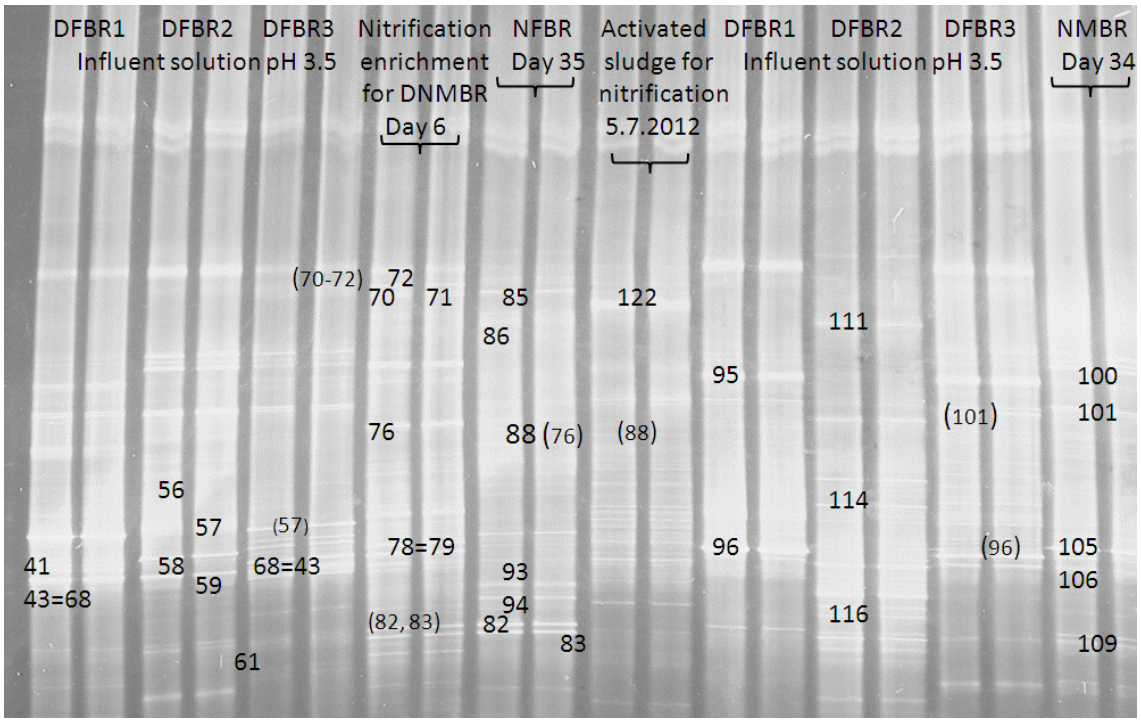

Figure 4.3.1. DGGE bands of from DFBR1-3, NMBR, NFBR and activated sludge used in nitrification reactors.

Appendix 1 and 2 presents sequenced partial 16S rRNA results from the numbered bands in the figure 4.3.1. Table 1 in Appendix 1 presents results from denitrification reactors and table 1 in Appendix 2 from nitrification reactors. Band numbers, their lengths as nucleotides, cultured strains most similar to the bands and their phylogenetic groups, similarities as matched nucleotides/ total nucleotides (%) and accession numbers are presented.

4.3.1. Denitrification

Results from previous study, from DFBR1-3, are presented in Appendix 3 (Papirio et al. 2013). At fifth day all DFBRs contained *Nitrospirae* strains (with 95-98% similarity to *Nitrospira mosciviensis)* and *β-proteobacteria strains* (with 99% similarity to *Ferribacterium limneticum and Zooloea caeni)*. In addition DFBR3 contained *Actinobacteria* (with 99-100% similarity to *Iamia majanohamensis*).

At days 123-147 (influent pH 7) DFBRs contained several *β-proteobacteria* strains (97-99% similarities to *Dechloromonas denitrificans, Dechloromonas hortensins* and *Azospira restricta*) and *N. mosciviensis. Z. caeni, F. limneticum* and *I. majanohamensis* weren't detected anymore.

At days 279-281 (influent pH 3.5), different *Dechloromonas* bacteria were detected from all DFBRs. In addition DFBR2 reactor contained one band of Δ-proteobacteria (99% similarity with *Geobacter thiogenes* and *Geobacter lovleyi)* and α-proteobacteria (98% similarity with *Rhodobacter vinaykumaraii, Catellibacterium and Haematobacter massiliensis*).

At days 342-343 (influent pH 2.5), DFBR1 and DFBR3 contained *Dechloromonas (β-Proteobacteria), Sulfirospirillum* and *Geospirillum* (*ε-proteobacteria, Campylobacteraceae)* strains. From DFBR2 only uncultured sequenced matched DGGE bands, these uncultured bacteria belonged to *Bacteroidetes, Firmucutes* and *Fibrobacteres*.

In summary, efficient enrichment of denitrifying microorganisms was obtained in all denitrification reactors. Microbial diversity was greater in room temperature than in 7°C.

4.3.2. Nitrification

Nitrification enrichment for DNMBR at day 6 contained *Trachelomonas volvocinopsis* var. *Spiralis (Euglenophyceae/ Euglenales), Ferruginibacter lapsinanis (Bacteroidetes/ Sphingobacteriia), Candidatus Nitrotoga* and *Candidatus Nitrospira* genera. NMBR at day 35 contained several uncultured strains and bacteria belonging to Dechloromonas and *Geobacter* genera.

From the original activated sludge for nitrification only *Ferruginibacter lapsinanis, Sediminibacterium salmoneum* (*Bacteroidetes, Sphingobacteriia*) and one uncultured bacterium could be recognized. However, DGGE band from sludge indicate the largest variety of different microorganism in our study. Bands were most probably too close to each other for proper separation and sequencing.

In summary, efficient enrichment of nitrifying microorganisms was obtained in all nitrification reactors. In addition some denitrifying microorganisms and photoautotrophic green sulphur bacteria were found.

5. DISCUSSION

5.1. Factors affecting denitrification

Yhis study demonstrated that pH 4.8 totally inhibited denitrification for denitrifiers originating from a neutral sludge of a municipal wastewater treatment plant. Similar pH effects have been reported in a pure culture study (Valera & Alexander 1961). Lowest pH limits for denitrification in soil and sediment studies were pH 2.9 (Parkin et al. 1985) and pH 2.6 (Baeseman et al. 2006). Based on theory of van den Heuvel et al. (2010) denitrification occurred at such low pH values because of the heterogenic structure of soils and sediments creating pH gradients. Microorganisms are also able to adapt to acidic conditions. This is supported by studies of Parkin et al. (1985) and Šimek et al. (2002), in which optimal pH values for denitrification were close to the original soil pH values, varying between pH 3.9 and 6.3. In the study of Baeseman et al. (2006) stream sediments were contaminated with acid mine drainage, resulting in a better resistance of the microorganisms to acidic conditions.

Even if denitrification has been proven to occur at low pH, reaction rates have shown to be slow under these conditions. Parkin et al. (1985) reported successful denitrification at pH 3.54 but with denitrification rates 30 times lower than at pH 6. In addition, low pH in soils seemed to decrease microbial diversity and create more intermediate products, including nitrous oxide. (Nägele & Conrad 1990; Blosl & Conrad 1992; Šimek et al. 2002; Baeseman et al. 2006). These results indicate that pH for denitrification should be kept above pH 6, to avoid the formation of undesirable intermediate products such as N_2O.

Even though limiting pH in batch assays was 4.8, the continuous FBRs neutralized the feed pH of 2.5. The buffered pH-water was recycled back diluting the acidic influent. Complete denitrification was observed both at 7°C and 21°C in FBRs. Efficient denitrification in 7°C has been reported also in the literature (Zaitsev et al. 2008; Vackova et al. 2011).

Excess of ethanol was found to be beneficial for denitrification. Stoichiometric ethanol/nitrate ratio resulted in incomplete denitrification. Doubling the stoichiometric ethanol/nitrate ratio, ethanol and nitrate were removed and nitrite did not accumulate even with a 5.4h HRT. Carbon source supplementation resulted from the presence of other ethanol-utilizing microorganisms and the use of ethanol as carbon source. Benefits of excess of carbon source (ratio between 2:1 - 4:1) for denitrification have been reported also in previous studies (Heylen et al. 2006; Panthi 2009). Results from pH batch bottle assay confirmed this result. At pH 5.3 addition of ethanol after 4.5 hours increased denitrification rate and led to complete denitrification. Addition of more

external carbon source increases costs, but it is essential for complete nitrate and nitrite removal.

Metal dissolution was not similar with all studied metals. Addition of 50 mg/l of copper, cobalt, nickel and arsenic to batch assays at pH 7 resulted in initial dissolved concentrations of 0.8, 55, 63 and 10.5 mg/l. With these concentrations denitrification rate was 11, 15, 18 and 34% slower, respectively, compared to similar experiments without metals. Arsenic was the most toxic compound considering added metal quantities. However, in arsenic batch assay pH was not controlled, so part of the inhibition could have also been due to higher pH environment. In summary, the toxicity of metals towards denitrification decreased in the following order As > Ni > Co > Cu.

Based on previous literature, all studied metals showed low inhibition on denitrification with relatively high soluble concentrations. This might result from nature of batch experiments. When cells are obtained from sources containing only low concentrations of metal, it takes some time before inhibitory concentrations of metals are uptaken into cells. During this initial period inhibitory effects of metals are low. This is supported by results of Hu et al. (2004) indicating higher toxic effect of metals in continuous flow reactors compared to batch assays. However, batch assays give important insight to metal toxicities.

5.1.1. Copper

In this study, copper was almost completely precipitated at pH 7. High precipitation is reported also in the literature (Cecen et al. 2009). All the soluble copper concentrations were close or below the detection limit of the AAS (0.5 mg/l).

Inhibition of 20, 40 and 80% was previously reported with soluble concentrations of 0.40, 0.95 and 2.50 mg/l (Ochoa-Herrera et al. 2011). In this study, denitrification rate was 11% inhibited with soluble concentrations of 0.77 mg/l. Higher inhibition was achieved with added 500 and 1000 mg/l of copper, even though soluble concentration were only 0.27 and 0.35 mg/l.

5.1.2. Arsenic

In arsenic batch assay results were obtained in experiments without pH control. Addition of phosphate could have affected arsenic toxicity, as phosphate closely resembles arsenate and can thus reduce arsenate uptake into the cells. However, increase of pH causes metal precipitation and might have reduced arsenic uptake as well. Because of the pH increase, results obtained are not directly comparable to other metal toxicity results.

Initial soluble arsenic concentrations of 3.5-13.9 mg/l, corresponding to 10-75 mg/l of added arsenic, resulted in 38-44% slower denitrification compared to batch assays without arsenic. Based on previous toxicity results from literature, arsenite, the most toxic form of arsenic, seems to be the most probable form. Panthi (2009) reported that arsenite concentrations of 5, 10 and 18 mg/l decreasead denitrification rate by 41, 57

and 79 % compared to experiment without arsenite. Denitrification rate with arsenate in the same study only decreased denitrification by 9.4, 15 and 30% with concentrations as high as 50, 100 and 2000 mg/l, respectively.

In the present study, arsenic affected ethanol consumption significantly less than nitrate removal. Ethanol removal was inhibited by 30 % after 9 hours showing higher sensitivity of denitrifiers to arsenic than other ethanol-oxidizing bacteria.

5.1.3. Nickel

Soluble nickel concentration decreased significantly between 1-3 hours. As pH remained stable, nickel was most probably uptaken into the biomass.

Initial soluble nickel concentrations of 12, 63 and 123 mg/l, corresponding to 10, 50 and 100 mg/l of added nickel, resulted in 4, 18 and 91% slower denitrification compared to batch assays without nickel. Higher soluble concentrations than the added ones, were most probably due to the inaccurancy in sample dilution or metal analysis.

In literature, Ni was shown to be toxic for denitrifiers even at low concentrations. Lawrence et al. (2004) reported negative effects on denitrification with Ni concentration as small as 0.05 mg / l.

5.1.4. Cobalt

Initial soluble cobalt concentrations of 0.57, 55 and 87 mg/l, corresponding to 0.5, 50 and 100 mg/l of added cobalt, resulted in 5, 15 and 26% slower denitrification compared to batch assays without cobalt. Ethanol removal inhibition was similar to denitrification inhibition. However, in literature cobalt toxicity was demonstrated at lower Co concentration. Sakadevan et al. (1999) found that even 5 µg cobalt/ g wetland sediment results in 39 % inhibition.

5.2. Nitrification in FBR and MBR

Different membrane types and mixing systems were compared in MBR systems. Hollow fiber membrane fouled slower than flat sheet membranes. Attachment of aeration pipes directly under hollow fibre membrane reduced membrane fouling. Mixing with continuous circulation resulted in sludge settling on the bottom of the reactor where continuous stirring with 120 rpm resulted in complete sludge suspension.

Both MBR and FBR systems showed effective nitrification at 21°C and pH range of 6-8.4 when 100 mg ammonium/l was continuously added with 20 h and 7 h HRTs, respectively. Only sludge settling in the beginning of NMBR experiment reduced nitrification.

Sum of total nitrate and nitrite yield as nitrogen in NMBR was significantly lower (average 53.7 % of the theoretical conversion from ammonium) than in NFBR (81.4%) and nitrification enrichment for DNMBR (81.1%). It was lower than expected, considering that almost all ammonium was oxidized. This might result from

simultaneous denitrification and nitrification in NMBR reactor. As a confirmation, microbial community analyses releaved the existence of denitrifying *Dechloromonas* strains in the NMBR at day 34. Long storage time of activated sludge in 4°C (50 days), before reactor start-up, could have enriched *Dechloromonas* strains, since *D. denitrificans* has been reported to grow even at temperatures as low as 5°C (Horn et al. 2005). In addition, in the beginning of NMBR experiment, sludge was partially settled in the bottom of the reactor, which might have enabled anoxic biofloc formation. This is supported by research of Li et al. (2008), in which denitrifying bioflocs were formed inside nitrifying bioreactor when denitrifyers were protected from frictions. In our study, after increase of sludge mixing (at 33 days) total nitrate and nitrite yield increased. However, it was not stable but varied between 38-95 % of theoretical maximum yield with average of 68.3%.

Simultaneous denitrification and nitrification is an useful processes in bioreactors for total nitrogen removal. It leads to lower reaction volumes and lower costs of chemicals and pumping. However, if all nitrate is not removed in the simultaneous process, a separate denitrification reactor is needed to complete nitrogen removal. For that denitrification reactor, concentration of remaining nitrate should be analyzed to evaluate need for additional carbons source and desing of operational parameters.

5.3. Simultaneous denitrification and nitrification in MBR

Control of aeration seemed to be the major parameter affecting the operation of a combined denitrification and nitrification reactor. This study showed that when aeration period was 60 minutes, elongation of non-aeration period from 20 minutes to 60 minutes enhanced denitrification. Nitrification decreased when the non-aeration time was extended, but recovered in a few days. However, further studies with stored DO data and optimization of the parameters are needed.

Both MBR and FBR systems showed effective nitrification at 21°C and pH range of 6-8.4 when 100 mg ammonium/l was continuously added with 20 h and 7 h HRTs, respectively. Sludge settling in the beginning of NMBR experiment seemed to reduce nitrification.

Sum of total nitrate and nitrite yield as nitrogen in NMBR was significantly lower (average 53.7 % of the theoretical conversion from ammonium) than in NFBR (81.4%) and nitrification enrichment for DNMBR (81.1%). It was lower than expected, considering that almost all ammonium was oxidized. This might result from simultaneous denitrification and nitrification in NMBR reactor. As a confirmation, microbial community analyses releaved the existence of denitrifying *Dechloromonas* strains in the NMBR at day 34. Long storage time of activated sludge in 4°C (50 days), prior to reactor start-up, could have enriched *Dechloromonas* strains, since *D. denitrificans* has been reported to grow even at temperatures as low as 5°C (Horn et al. 2005). In addition, in the beginning of NMBR experiment, sludge was partially settled to the bottom of the reactor, which might have enabled anoxic biofloc formation. This is

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5.4. Microbial communities

5.4.1. Denitrification

DFBR2 contained larger variety of different strains than DFBR1 and DFBR3. Most denitrifying microorganisms grow optimally up at 20-40°C (Stolz et al. 1999; Horn et al. 2005; Wolterink et al. 2005; Quan et al. 2006). Thus low temperature of only 7°C in DFBR1 could support the growth of only few, psycrotrophic denitrifiers. In DFBR3, efficiently growing denitrifyers were enriched, since activated carbon carrier and biomass were several times partially removed from DFBR3 for the batch bottle assays. With influent of pH 2.5, only few bands could be sequenced from DFBR2. Those bands showed similarity only to uncultured *Bacteroidetes, Firmicutes* and *Fibrobacteres* strains.

After enrichment of 123 days with neutral influent solution, main denitrifiers in DFBR1 and DFBR3 were *Dechloromonas* strains. Closer comparison to GenBank strains showed that these strains were either unidentified or matched several different genera. Some samples matched *Dechloromonas denitrificans* and *Dechloromonas hortensins*, some *D. hortensis, Dechloromonas aromatica* and *Ferrobacterium limneticum*.

D. denitrificans grows at 5-35°C and pH 6.1-8.3 (Horn et al. 2005), which supports its existence in all the DFBRs. Limiting growth temperatures of *D. hortensis* (genus that matched most of the *Dechloromonas* strains in this study) were not found from the literature, but its optimal conditions of 30°C and pH 7.2 (Wolterink et al. 2005), resembled those of *D. denitrificans*. However, presence of these two genera cannot be confirmed as Wolterink et al. (2005) and Horn et al. (2005) have reported that *D. denitrificans* and *D. hortensis* do not grow on ethanol. Existence of some other, closely related *Dechloromonas* genera, seems thus more propable.

Sulphurospirillum strains were found from DFBR1 and DFBR3 when pH of the influent solution was decreased from 3.5 to 2.5. They belong to *ε-proteobacteria*, which are able to use nitrate as terminal electron acceptor. Three different genera of *Sulphospirillum* showed 99% similarities to our samples: *Sulfurospirillum arsenophilum, Sulfurospirillum halorespirans* and *Sulfurospirillum multivorans. Sulfurospirillum barnesii* matched only with 97% similarity. *S. arsenophilum* grows optimally at 20°C and in pH 7.5 and is able to convert nitrate to ammonium (Stolz et al. 1999). *S. barnesii* has higher optimal growth temperature of 33°C in same the pH of 7.5

(Stolz et al. 1999). *S. halorespirans* grow optimally at 25-30°C (Luijten et al. 2003) and *S. multivorans* at 30°C in pH of 7-7.5 (Scholz-Muramatsu et al. 1995).

Rhodobacter species, found in DFBR2 on day 279, are common denitrifiers (Zumft 1997, p.592). Four different *Rhodobacter* genera matched strain number 59. Presence of *Rhodobacter vinaykumaraii*, which was found with 98% similarity to this strain, does not seem probable, since Horn et al. (2005) reported that *R. Vinaykumaraii* requires 1-4 % (w/v) NaCl concentration and cannot use ethanol as an electron donor. Also presence of *Haematobacter massiliensis* and *Catellibacterium*, which matched the strain with 98% similarity, are not likely, since they have been reported to require aerobic conditions (Tanaka et al. 2004a; Helsel et al. 2007). The fourth strain was defined only as a *Rhodobacter.*

Few non-denitrifying microorganisms were found also. *Nitrospirae moscoviensis,* detected on day 5 in all the DFBRs, is an autotrophic nitrifying microorganism oxidizing nitrite to nitrate (Daims 2001). Even though nitrite is produced as an intermediate in denitrification process, lack of oxygen and presence of faster growing heterotrophs inhibited its growth later in DFBRs. *Geobacter thiogenesis* and *Geobacter lovley* were found in DFBR2 when influent solution of pH 3.5 was used. These genera belong to *Δ-proteobacteria* phylum. As members of *Geobacteracea* family, they are capable of Fe(III), S° and fumarate reduction. *G. thiogenesis* is also able to grow with reductive dehalogenation, optimally at 30°C temperature and pH near 7.0 (Nevin et al. 2007). *G. lovleyi* has reported to reduce uranium (Amos et al. 2007).

5.4.2. Nitrification

The same activated sludge was used for seeding all nitrification reactors. Since all the experiments were not started at the same time, sludge was stored in 4°C, resulting in some changes to the original microbial community. Other changes resulted from the enrichment in reactors.

Nitrification enrichment for DNMBR and NFBR showed similar microbial communities. The main nitrifying microorganism in both reactors was *Candidatus Nitrospira defluvii.* It is an aerobic and chemolithoautotrophic nitrite oxidizing bacteria (Kostan et al. 2010) and one of the most abundant nitrifiers in natural environments (Horn et al. 2005) and wastewater treatmen plants (Daims et al. 2001). NFBR contained also *Terrimonas lutea* strains. *T. lutea* is strictly aerobic and grows optimally at tempartures of 10-37°C. It is reported to reduce nitrate. (Xie & Yokota 2006). Both reactors contained also non-nitrifying organisms, including *Ferruginibacter lapsinanis.* NFBR cultures were also formed by *Sediminibacterium salmoneum* strains, which are not reported to reduce nitrate (Qu & Yuan 2008; Lim et al. 2009). *F. lapsinasis* is a strictly aerobic bacterium with optimal growth at 18-30°C temperatures and pH 6-8 (Lim et al. 2009). *S. salmoneum* grows at 18-37°C temperatures and in pH 6-7.5, optimally at 22-28°C and at pH 7.0 (Qu & Yuan 2008). They both belong to family of *Chitinophagacea*, together with nitrate reducing *T. lutea.*

Microbial community in NMBR on day 34 differed from the enrichment for DNMBR and NFBR. Two denitrifying *Dechloromonas* strains, one *Geobacter* strain and three uncultured strains were found. One uncultured strain was indentified as a *Chlorobi* or *Ignavibacterium*. *Chlorobi* bacteria are obligately anaerobic photoautotrophs, which can oxidice sulfur compounds, H_2 or ferrous iron and fix carbon (Bryant & Frigaard 2006). They include green sulphur bacteria. Green colour of the reactor can be seen in the photo of figure 3.4.3.2.A. *Chlorobi* bacteria have been found also from other nitrification processes where microorganisms originated from wastewater treatment plants (Li et al. 2009; Cho et al. 2011).

6. CONCLUSIONS

Present study reports parameters that have to be considered in biological nitrogen removal process for mining wastewaters. The microorganisms originating from municipal wastewater treatmen plant included denitrifying *Dechloromonas*, *Sulphurospirillum* and *Rhodobacter* species and nitrifying *Candidatus Nitrospira defluvii.*

Limiting pH for denitrification in batch assays was pH 4.8 based on batch assays. Denitrification process in continuous fluidized bed reactors (FBRs) was able to neutralize influent solution of pH 2.5 due to produced alkalinity. Denitrification was complete at 7°C and 21°C temperatures, when double stoichiometric ethanol/nitrate ratio was provided for the cells.

All studied heavy metals reduced denitrification in batch assays. When 50 mg/l of copper, cobalt, nickel and arsenic were added their soluble concentrations were 0.8, 55, 63 and 10.5 mg/l and denitrification rate decreased 11, 15, 18 and 34%, respectively. Added nickel concentration of 100 mg/l resulted in 91% slower denitrification. In summary, the toxicity of metals towards denitrification increased in the following order As > Ni > Co > Cu.

Both FBR and membrane bioreactor (MBR) were suitable for nitrification. Complete nitrification was obtained at 21°C when pH was maintained at 6-8.4 and 100 mg ammonium/l was continuously added with 7 h and 20 h hydraulic retention times (HRTs), respectively. In MBR continuous stirring with 120 rpm and use of aeration directly under hollow fiber membranes guaranteed sufficient sludge suspension and prevented membrane fouling.

Average total nitrogen removal in simultaneous nitrification and denitrification reactor was 47%. When operated with 60 minutes aeration and 20-60 minutes non-aeration intervals, highest total removal of 67% was achieved at 60 minutes non-aeration interval was used.

In summary, the present study shows promising results for biological nitrogen removal from mining wastewaters in bioreactors. Denitrification is possible at 7°C, even with influent solution of pH 2.8. Two types of bioreactors, FBR and MBR, are able to maintain sufficient biocumminity of slowly growing nitrifyers for efficient ammonium removal. Both denitrifying and nitrifying microorganisms can be easily enriched from municipal wastewater treatment plants. In batch experiments low concentrations of common heavymetals Ni, Co, Cu and As do not inhibite denitrification remarkably.

7. RECOMMENDATIONS FOR FUTURE RESEARCH

Previous publications have reported increased N_2O production in low pH and copper containing studies, whilst in this study no gaseus products were investigated. This is however essential to prevent N_2O formation and to understand reaction mechanisms better.

It would be important to study metal toxicity in continuous flow reactors. This way cells would have enough time to uptake toxic metals and the actual toxic effects on bacteria could be verified. In addition, several different metals should be tested at the same time, since mining wastewaters contain multiple metals and their combined effects can be significantly different from effects of one single metal.

So far, no differences were found within the DFBRs operated at 7°C and 21°C. In previous studies, denitrification occurred slower at low temperatures. Therefore, using 10 mg of VSS in 1,1 liter reactor, nitrate loading rate could be increased from the present 870 mg NO_3^-/l/d in order to observe differences between the two operating temperatures.

In this study, simultaneous nitrification and denitrification process in single reactor was not as efficient as separate nitrification and denitrification reactors. However, longer enrichment of the bacteria and further optimization of aeration intervals could enhance nitrogen removal.

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APPENDIX 1: Microbial community in denitrification reactors

Table 1. Cultured strains most similar to sequenced DGGE samples from DFBR1-3. Sample name and sampling day, band number and length as nucleotides, similar strains with phylogenetic groups (α = α –Proteobacteria, β = β-Proteobacteria, Δ = Δ-Proteobacteria, ε= ε-Proteobacteria), similarity as matched nucleotides/ total nucleotides % (Sim) and accession number.

Sample	Band (lenght)	Cultured strain most similar to query sequence	Phylogenetic group	Sim	Accession number
DFBR1 influent pH 3.5	41 (413)	*Dechloromonas*	*β/Rhodocyclales*	98	gi|9937338|AF288775.1
		Azovibrio restrictus		97	gi|265678376|NR_028678.1
	43 (461) =68	*Dechloromonas*		99	gi|148748886|EF632559.1
		Dechloromonas hortensis		99	gi|343202496|NR_042819.1
		Dechloromonas denitrificans		99	gi|343201364|NR_042090.1
DFBR1 influent pH 2.5	95 (453)	*Sulfurospirillum arsenophilum*	*ε/ Campylobacteraceae*	99	gi|343206214|NR_044806.1
		Sulfurospirillum halorespirans		99	gi|265678468|NR_028771.1
		Geospirillium		99	gi|7710967|Y18254.1
		Sulfurospirillum multivorans		99	gi|343206276|NR_044868.1
		Sulfurospirillum barnesii		97	gi|390192281|CP003333.1
	96 (501)	*Dechloromonas aromatica*	*β/Rhodocyclales*	98	gi|71845263|CP000089.1
		Ferribacterium limneticum		98	gi|219846872|NR_026464.1
		Dechloromonas		98	gi|45269130|AY084087.2
		Dechloromonas hortensis		98	gi|343202496|NR_042819.1
DFBR2 influent pH 3.5	57 (459) 56 (457) 61 (475)	*Geobacter thiogenes*	*Δ/ Desulfuromonadales*	99	gi|265678472|NR_028775.1
		Geobacter lovleyi		99	gi|365268871|JN982204.1
	59 (418)	*Rhodobacter*	*α/Rhodobacterales*	98	gi|304656610|FN995209.1
		Rhodobacter vinaykumaraii		98	gi|138753490|AM600642.1
		Catellibacterium		98	gi|395628215|JX046043.1
		Haematobacter massiliensis		98	gi|395132667|JQ958833.1
	58 (496)	*Dechloromonas*	*β/Rhodocyclales*	99	gi|374857917|AB696861.1
DFBR2 influent pH 2.5	111 (444)	Uncultured *Flavobacteria*	*Bacteroidetes*	99	gi|151506315|EF651645.1
		Uncultured *Sphingobacteriales*		98	gi|285307172|AM940674.1
		Uncultured *Chitinophaga*		98	gi|285307394|AM940896.1
	114 (443)	Uncultured Bacteroidetes		99	gi|62633634|AY902708.1
	116 (465)	Uncultured Firmicutes	*Firmicutes*	98	gi|395455782|HE573219.1
		Uncultured Fibrobacter	*Fibrobacteres*	98	gi|284027923|GU323642.1
DFBR3 influent pH 3.5	68 (464) = 43	*Dechloromonas*	*β/Rhodocyclales*	99	gi|148748886|EF632559.1
		Dechloromonas hortensis		99	gi|343202496|NR_042819.1
		Dechloromonas denitrificans		99	gi|343201364|NR_042090.1
DFBR3 influent pH 2.5	95 (453)	*Sulfurospirillum arsenophilum*	*ε/ Campylobacteraceae*	99	gi|343206214|NR_044806.1
		Sulfurospirillum halorespirans		99	gi|265678468|NR_028771.1
		Geospirillium		99	gi|7710967|Y18254.1
		Sulfurospirillum multivorans		99	gi|343206276|NR_044868.1
		Sulfurospirillum barnesii		97	gi|390192281|CP003333.1
	96 (501)	*Dechloromonas aromatica*	*β/Rhodocyclales*	98	gi|71845263|CP000089.1
		Ferribacterium limneticum		98	gi|219846872|NR_026464.1
		Dechloromonas		98	gi|45269130|AY084087.2
		Dechloromonas hortensis		98	gi|343202496|NR_042819.1

APPENDIX 2: MICROBIAL COMMUNITY IN NITRIFICATION REACTORS

Table 1. Cultured strains most similar to sequenced DGGE samples from NMBR1, NMBR2, NFBR and original activated sludge. Sample name and sampling day, band number and length as nucleotides, similar strains with phylogenetic groups (β = β-Proteobacteria, Δ = Δ-Proteobacteria), similarity as matched nucleotides/ total nucleotides % (Sim) and accession number.

Sample	Band (lenght)	Cultured strain most similar to query sequence	Phylogenetic group	Sim	Accession number
NFBR day 35	82 (479), 83 (479), 93 (437), 94 (483)	Candidatus Nitrospira defluvii	*Nitrospirae*	100	gi|300603458|FP929003.1
	70 (455), 71 (458)	*Trachelomonas volvocinopsis var. spiralis*	*Euglenophyceae/ Euglenales*	99	gi|261362019|FJ719709.1
	72 (469)	*Ferruginibacter lapsinanis*	*Bacteroidetes/ Sphingobacteriia/ Chitinophagaceae*	98	gi|343199116|NR_044589.1
	85 (459)	*Terrimonas lutea*		97	gi|343200563|NR_041250.1
	86 (320)	*Sediminibacterium salmoneum*		98	gi|359803058|AB682145.1
		Flavobacteria bacterium	*Flavobacteria/ Flavobacteriales*	98	gi|125860564|AB269814.1
	88 (382)	*Uncultured bacterium*		97	gi|387308250|JQ791674.1
Nitrification enrichment for DNMBR day 6	70 (455), 71 (458)	*Trachelomonas volvocinopsis var. spiralis*	*Euglenophyceae/ Euglenales*	99	gi|261362019|FJ719709.1
	72 (469)	*Ferruginibacter lapsinanis*	*Bacteroidetes/ Sphingobacteriia*	98	gi|343199116|NR_044589.1
	76 (447)	Uncultured *Bacteroidetes*		99	gi|237947412|CU924663.1
	78 (391), 79 (452)	*Candidatus Nitrotoga arctica*	*β-proteobacteria*	99	gi|144984867|DQ839562.1
		Candidatus Nitrotoga		99	gi|222432101|FJ263061.1
	82 (479), 83 (479)	*Candidatus Nitrospira defluvii*	*Nitrospirae*	100	gi|300603458|FP929003.1
NMBR day 34	100 (506)	Uncultured *bacterium*		99	gi|304366592|HQ010810.1
	100 (482)	Uncultured *Ignavibacterium*		98	gi|343174647|JN217054.1
		Uncultured *Chlorobi bacterium*		98	gi|242346470|GQ183427.1
	101 (466)	Uncultured *Sphingobacteriales bacterium*		98	gi|391882714|JQ723668.1
	105 (494)	*Dechloromonas*	*β/Rhodocyclales*	99	gi|374857917|AB696861.1
		Dechloromonas agitata		98	gi|219857253|NR_024884.1
		Dechloromonas hortensis		98	gi|343202496|NR_042819.1
		Azovibrio restrictus		98	gi|265678376|NR_028678.1
		Azonexus hydrophilus		97	gi|121484280|EF158391.1
		Azonexus caeni		97	gi|343200330|NR_041017.1
		Ferribacterium limneticum		97	gi|219846872|NR_026464.1
	106 (440)	*Dechloromonas*		97	gi|374857917|AB696861.1
	56, 57, 61, 109 (446)	*Geobacter thiogenes*	*Δ/Desulfuromonadales*	98	gi|265678472|NR_028775.1
		Geobacter lovleyi		98	gi|365268876|JN982209.1
Activated sludge for nitrification	72 (469)	*Ferruginibacter lapsinanis*	*Bacteroidetes/ Sphingobacteriia*	98	gi|343199116|NR_044589.1
	122 (439)	*Sediminibacterium salmoneum*		99	gi|343205781|NR_044197.1
	88 (382)	*Uncultured bacterium*		97	gi|387308250|JQ791674.1

APPENDIX 3: MICROBIAL COMMUNITY RESULTS FROM PREVIOUS STUDY (PAPIRIO ET AL. 2013)

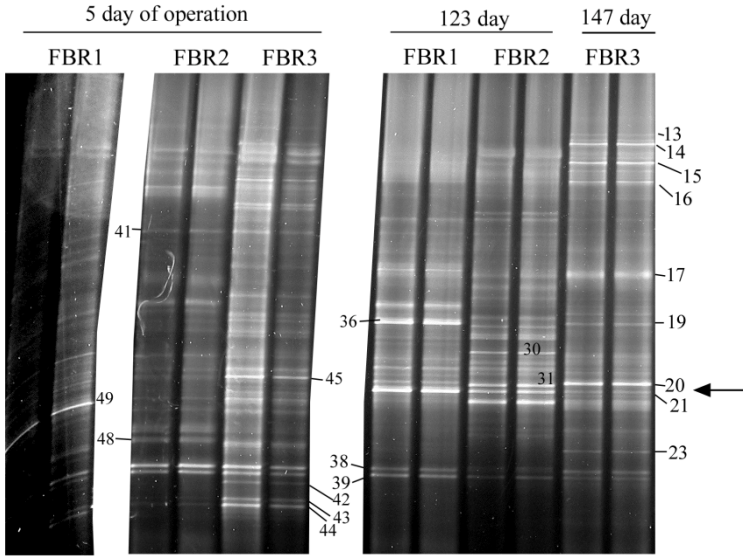

Figure 1. DGGE bands of microorganisms detected in previous studies from DFBR1-2. (Papirio et al. 2013, *fig.5*)

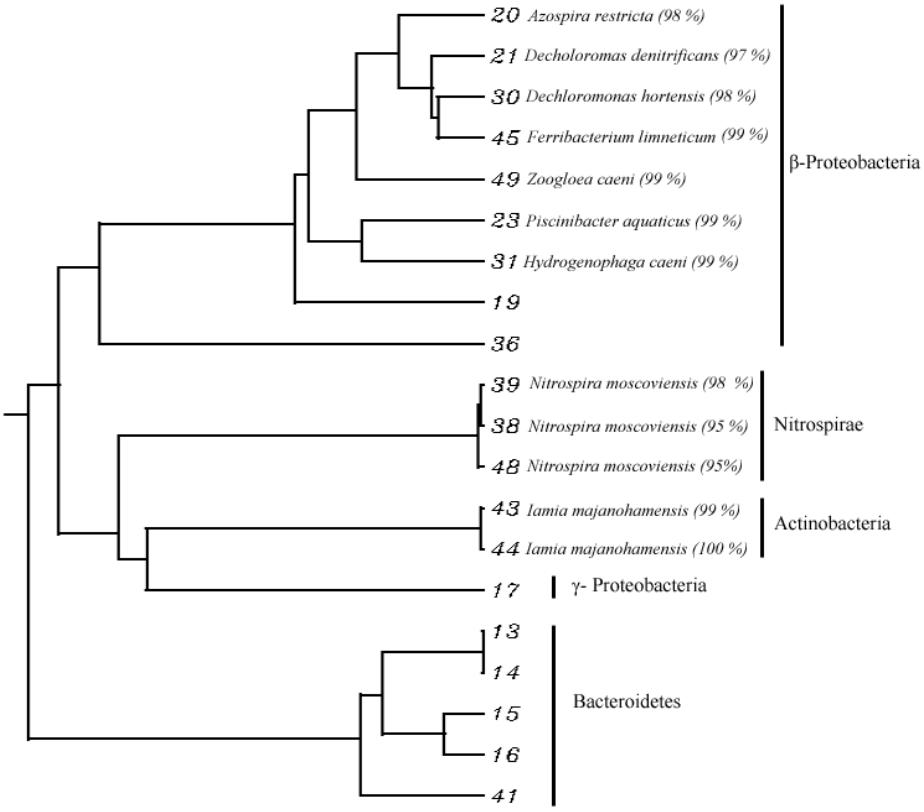

Figure 2. Dendogram of relationships of microorganisms detected in previous studies from DFBR1-3. (Papirio et al. 2013, fig.6)

APPENDIX 4: CHEMICALS

Table 1. Used chemicals and their producers.

Chemical	Producer
$CaCl_2 \cdot 2H_2O$	Merck, Germany
$CaCO_3$	
$CoCl_2 \cdot 6H_2O$	Merck, Germany
Ethanol (99,5 % Etax Aa)	Altia, Finland
KH_2PO_4	J. T. Baker, Holland
$MgCl_2 \cdot 6H_2O$	Merck, Germany
$MnCl_2 \cdot 4H_2O$	Merck, Germany
$NaHCO_3$	Merck, Germany
NH_4Cl	Merck, Germany
$Na_2MoO_4 \cdot 2H_2O$	J. T. Baker, Holland
$NaNO_3$	Merck, Germany
$NiCl_2 \cdot H_2O$	Merck, Germany
$CuCl_2 \cdot H_2O$	Merck, Germany
$NaAsO_2$	Aldrich
$Na_2HPO_4 \cdot H_2O$	Merck, Germany
Na_2HPO_4	Merck, Germany
HNO_3	J. T. Baker
BSA (Bovin Serum Albumin)	Fermentas, USA
6X DNA Loading dye	Fermentas, USA
DreamTaq Buffer	Fermentas, USA
dNTP mix	Fermentas, USA
Ba357 F-GC reverse primer	Thermo Scientific, Germany
907r foward primer	Thermo Scientific, Germany
Nuclease free water	Thermo Scientific, Germany
Sybr (R) safe DNA gel stain	Invitrogen, Life tehchonologies