Microbial nitrate removal from mining waters

Maria Hellman
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

Licentiate thesis
Swedish University of Agricultural Sciences
Uppsala 2018

Cover:
Anaerobic nitrogen transformation pathways.
(Illustration: Maria Hellman)

ISBN (print version) 978-91-576-9589-5
ISBN (electronic version) 978-91-576-9590-1

Print: SLU Service/Repro, Uppsala 2018

Microbial nitrate removal from mining waters

Abstract

Mining activities cause high levels of nitrogen in the process water. The nitrogen originates from the blasting procedure, where undetonated explosives in the form of ammonium nitrate dissolve in the water. Part of the water is discharged to receiving water bodies where it may cause environmental problems. To meet current regulations, the nitrate concentration in the discharge water needs to be reduced. Two nitrate removal systems were studied in the thesis, denitrifying bioreactors and pond sediments. The overall aim was to identify and investigate factors affecting microbial nitrate removal in the systems. This was done by analysing the microbial communities in the systems using molecular methods and biochemically by measuring denitrification rates.

We reported for the first time a pilot-scale denitrifying bioreactor treating mining water. The reactor efficiently removed nitrate after addition of external carbon. There were indications on preferential flow paths in the reactor, hence probably only a part of the substrate volume contributed to the removal. In a laboratory experiment we tested different reactor substrates; barley straw and *Carex rostrata* supported higher nitrate removal rates than woodchips did. Initially, there was an increase in bacterial alpha-diversity in all reactor types and when the bacterial community stabilised, it was reflected in more stable nitrate removal rates, most obvious in the woodchip reactors. All three substrates developed distinct bacterial communities. The denitrification rates in pond sediments from the LKAB mining site in Kiruna, Sweden were limited by organic carbon availability. A microcosm experiment showed that treating the sediment with carbon for a period can increase the rates. However, the choice of carbon type impacts the metabolic pathways in the system and adverse effects in the form of methane production and accumulation of ammonium were observed in the treatment with carbon in the form of algae.

To conclude, constructed systems where denitrification takes place can successfully remove nitrate from mining waters. Organic carbon quality and availability is a crucial factor for the removal efficiency and for determining the composition of the microbial communities performing the reduction of nitrate.

Keywords: denitrification, passive system, denitrifying bioreactor, sediment, microbial community

Author's address: Maria Hellman, SLU, Department of Forest Mycology and Plant Pathology,
P.O. Box 7026, 750 07 Uppsala, Sweden.
E-mail: maria.hellman@slu.se

Mikrobiell reduktion av nitrat från gruvvatten

Sammanfattning

Gruvdrift orsakar höga halter av kväve i det vatten som cirkuleras i processen. Kvävet kommer från arbetet med att spränga; de sprängämnen som inte detonerar löses upp i vattnet i form av ammoniumnitrat. En del av vattnet släpps ut i de omgivande vattendragen där det kan orsaka miljöproblem. För att uppfylla gällande bestämmelser måste nitratkoncentrationerna i utsläppsvattnet minskas. I avhandlingen studeras två system för att reducera nitrathalterna, denitrifierande bioreaktorer och dammsediment. Avhandlingens övergripande mål var att identifiera och studera faktorer som påverkar mikrobiell nitratreducering i systemen genom att göra biokemiska och mikrobiologiska analyser. Vi använde molekylära metoder för att studera hur mikrobsamhällena i systemen var sammansatta och denitrifikations-hastigheten användes som ett mått på den biokemiska aktiviteten. Vi beskrev för första gången en denitrifierande bioreaktor som reducerade nitrathalten i gruvvatten. Reaktorn avlägsnade nitrat ur vattnet effektivt, men först efter tillsats av organiskt kol. Flera av resultaten tydde på att det fanns olika flödesvägar genom reaktorn, följaktligen bidrog inte hela reaktorvolymen till nitratreduktionen. Tre möjliga substrat för bioreaktorer testades i ett laboratorieförsök; strå av korn och starren *Carex rostrata* reducerade nitrathalten mer effektivt än vad träflis gjorde. I början av experimentet ökade den bakteriella alfa-diversiteten i alla reaktortyper och när ökningen planade ut reflekterades det i nitratreduceringen, tydligast i träsflisreaktorerna. Unika bakteriesamhällen utvecklades i alla tre substrat. Tillgängligheten av organiskt kol begränsade denitrifikationen i dammsediment från LKAB:s gruvområde i Kiruna. Ett annat försök visade att en periods kol-tillsatser till sedimentet kan öka denitrifikationen. Valet av koltyp påverkar vilka metabola vägar som uttrycks i systemet och vi kunde se negativa effekter i form av metanproduktion och anrikning av ammonium i vattnet när kol tillsattes i form av alger. Sammanfattningsvis, konstruerade system där denitrifikation äger rum kan avlägsna nitrat från gruvvatten. Typen av och tillgången till organiskt kol är en avgörande faktor för systemens effektivitet och för vilka specifika mikrobsamhällen som utvecklas i dem.

Nyckelord: denitrifikation, passiva system, denitrifierande bioreaktor, sediment, mikrobiellt samhälle

Författarens adress: Maria Hellman, SLU, Institutionen för skoglig mykologi och växtpatologi
Box 7026, 750 07 Uppsala, Sweden.
E-mail: maria.hellman@slu.se

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Herbert, R.B.*, Winbjörk, H., Hellman, M. and Hallin, S. (2014). Nitrogen removal and spatial distribution of denitrifier and anammox communities in a bioreactor for mine drainage treatment. *Water Research*, 66, pp. 350-360.

II Hellman, M. *, Hubalek, V., Almstrand, R., Peura, S. and Hallin, S. Substrate type determines activity and community composition in lab-scale bioreactors for nitrate removal by denitrification at low temperatures. (manuscript)

III Hellman, M., Bonilla-Rosso, G., Widerlund, A., Juhanson, J. and Hallin, S.* External carbon addition for enhancing denitrification modifies community composition and affects CH_4 and N_2O emissions in sub-arctic mining pond sediments. (manuscript)

* Corresponding author.

The contribution of Maria Hellman to the papers included in this thesis was as follows:

I Planned and took part in the sampling. Modified the DNA extraction method for the sample type “gravel mixed with sawdust”. Performed the qPCR analyses. Contributed to the writing of the manuscript.

II Contributed to the idea and design of the experiment and to the planning of monitoring and sampling. Did a major part of the monitoring, samplings and analyses. Analysed all but the sequence data. Wrote the manuscript with support from the co-authors.

III Contributed to the idea and design of the experiment. Planned all field and laboratory work. Did all field work together with G. B-R. Did almost all of the work in the laboratory. Analysed the data. Wrote the manuscript with support from the co-authors.

1 Introduction

For thousands of years, man has used metals for adornments, tools and weapons. During the Bronze Age, man developed skills to process ore and work metal. The Bronze Age was followed by the Iron Age, in northern Europe around 500 BC, and iron was extracted from pieces of bog ore found in bogs and lakes. It is believed that it took until 1100 AD before ore from bedrock could be used as a source for metals.

Traditionally, the rock was cracked with the help of heat from fires, and the first evidence of using explosives is from 1627 in Slovakia (Heiss and Oeggl, 2008). In modern mining, ANFO (ammonium nitrate mixed with fuel oil) is the most commonly used explosive (Forsyth et al., 1995). Ammonium nitrate is easily dissolved in water and incomplete detonation of the explosives as well as spillage during handling results in reactive nitrogen (N) in ground water, process water and in leachates from waste-rock dumps. Eventually, part of the water is discharged and may have negative environmental impacts in the receiving waters. This is of special concern since primary production in many lakes in high latitude regions, where many mines are located, is typically limited by nitrogen in those areas (Bergström et al., 2013). High levels of nitrogen thus cause eutrophication and in addition, both ammonium and nitrite are toxic to water-living organisms (Camargo and Alonso, 2006). It has been shown that mining activities do elevate total nitrogen concentrations in waters receiving mining effluents (Chlot et al., 2013).

Current water legislation in Sweden is under the European Water Framework Directive, which states that all lakes greater than 0.5 km^2 must show at least good ecological status by 2021 (EU Commission 2000). The major reason for many surface waters not currently reaching good ecological status in Sweden is eutrophication. To avoid costly restoration of water bodies, mining companies need to find effective solutions for mitigating the nitrogen concentrations in the discharge water.

There are a number of technics for nitrogen removal from water. Physical and chemical methods are available (Jermakka et al., 2015), but the majority of them are not suitable for mining effluents. Instead, biological methods where microorganisms convert the soluble nitrogen species to gaseous compounds are more suitable ways of removing the nitrogen. In order to be economically feasible for large volumes of water, industry is interested in so called passive or semi-passive solutions, i.e. processes with no or only little input in the form of energy or material once the system is built and in use. Thus, there is a need to develop other solutions than the traditional nitrogen removal processes used for example in wastewater treatment plants.

1.1 Aims and objectives

This thesis is part of the Vinnova project miNing where the overall objective was to identify and investigate passive or semi-passive treatment systems for mine discharge water. The systems should, when implemented in full scale, remove enough nitrogen for the receiving waters to have nitrogen concentrations in compliance with national and international legislation. To address the aim, I have studied two systems for microbial nitrogen removal, denitrifying cellulose-based bioreactors and the existing tailings ponds at a mining site. If nothing else is stated, "bioreactor" and "reactor" refers to denitrifying cellulose-based bioreactors in the following text. The specific objectives in the studies included in this thesis were to:

Paper I.
In a field pilot-scale reactor for nitrate removal:

i) determine the treatment capacity.
ii) investigate if there were preferential flow paths.
iii) determine the distribution and abundances of denitrifying and anammox bacterial communities.

Paper II.
In a laboratory experiment:

i) evaluate three cellulose-based substrates for their suitability as electron donors for denitrifying bioreactors at low temperature.
ii) analyse the development of microbial community compositions in the reactors in relation to nitrate removal rates.

Paper III.

In a field study and in a laboratory experiment:

i) investigate the denitrifying capacity and microbial community compositions in the sediments of the tailings and clarification ponds at a mine site.

ii) determine if the denitrification rates in the sediments could be enhanced.

iii) investigate which functional groups and community members that were dominating in the sediments after enhancement.

iv) monitor potential negative effects from the treatments aiming at enhancing denitrification.

2 Microbial nitrogen transformations and methods used

Nitrogen can have seven different oxidation states, from +5 in nitrate to -3 in ammonia, ammonium and when bound in organic compounds, giving the opportunity for numerous transformations between the different states. Although abiotic photo- and thermochemical reactions are observed, the majority of the nitrogen transforming reactions are mediated by microorganisms. The so far known microbial transformations in the biogeochemical nitrogen cycle cannot be described using a "cycle", they are reactions connected in a network, where many of the reactive compounds can take several directions (Kuypers et al., 2018). To further complicate the picture, nitrogen is also assimilated and incorporated into biomass by all organisms and subsequently mineralized to ammonium during decay.

2.1 Anaerobic nitrate removal

There are several microbial reactions that permanently remove nitrogen from water by reducing nitrate. These occur mainly under anoxic conditions. The starting point is the reduction of nitrate to nitrite, and the nitrite can then take two routes leading to three different nitrogen-transforming pathways: denitrification, anaerobic ammonium oxidation (anammox) or dissimilatory nitrite reduction to ammonium (DNRA; Fig.1). The first two processes end with gaseous nitrogen compounds, whereas DNRA leads to the production of ammonium. From a nitrogen removal perspective, this is a critical point for the fate of nitrate; will it be retained in the water in the form of ammonium or will it leave in the form of a gas? However, during denitrification the gases nitric oxide (NO) and nitrous oxide (N_2O) can be produced. Thus, even if the nitrogen is removed from the water phase, it can still have a negative environmental

impact as N_2O is a powerful greenhouse gas and NO contributes to acid rain and depletion of the ozone layer.

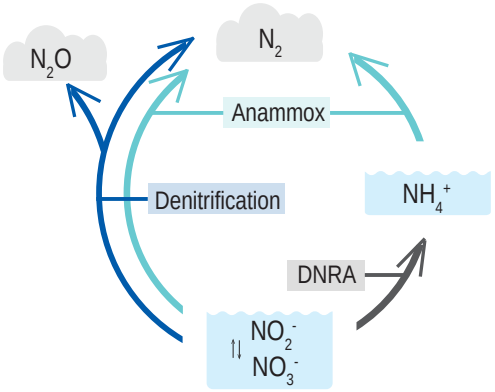

Figure 1. Reactants and end-products in anaerobic nitrogen transformation pathways.

Denitrification

Denitrification is an anaerobic respiratory pathway in which soluble nitrate or nitrite is stepwise reduced to gaseous compounds (Fig. 2a). The process is a facultative trait, triggered by low oxygen tension and the availability of a nitrogen oxide that can serve as electron acceptor, although aerobic denitrification has been observed for some bacteria (Zumft, 1997). Since nitrogen oxides are less effective electron acceptors than oxygen, meaning less energy is conserved, denitrification is down regulated in the presence of oxygen (Chen and Strous, 2013) and there are so far no known bacteria that use denitrification as the sole means of conserving energy in the form of ATP (Shapleigh, 2013). Microorganisms capable of performing all four steps from nitrate to dinitrogen gas (N_2) have sometimes been referred to as canonical denitrifiers (Stein and Klotz, 2016). However, with increasing knowledge it has become clear that the pathway is modular, i.e. microorganisms harbour different sets of the genes encoding for the enzymes needed for catalysing the full reaction (Graf et al., 2014; Zumft, 1997) and complete denitrification by a single organism might be the exception rather than the rule (Kuypers et al., 2018).

Denitrifiers are found in nearly all environments that receive oxygen to some extent (Shapleigh, 2013) and there are denitrifiers in all three domains of life. Most of the known bacterial denitrifiers belong to the Proteobacteria (Shapleigh, 2013; Philippot et al., 2007), but there are also members in a number of other phyla, for example in Firmicutes and Bacteroidetes (Graf et al., 2014). In the domain Eukarya, denitrification has been described in fungi (Maeda et al., 2015; Shoun et al., 1992) and in Foraminifera (Risgaard-Petersen et al., 2006; Woehle

et al., 2018). The overall reaction for denitrification with organic carbon (C) as the electron acceptor is depicted below.

$$4\ NO_3^- + 5\ CH_2O \rightarrow 2\ N_2 + 4\ HCO_3^- + H_2CO_3 + H_2O$$

The first step, reduction of nitrate to nitrite is a widespread trait (Kuypers et al., 2018) and not restricted to denitrifiers since the nitrite produced can be used also in other pathways (Stein and Klotz, 2016) or not be further reduced at all. In denitrifiers, the two most common nitrate reductases catalysing this step are the cytoplasmic NAR and the membrane bound NAP, encoded by the genes *narG* and *napA* respectively. The reaction is most often coupled to oxidation of an organic compound, but inorganic molecules as sulphur compounds or metals can also act as reducing agents (Hayakawa et al., 2013; Zhu and Getting, 2012). Abiotic reduction of nitrate to nitrite can occur as well, for example due to photodecomposition (Warneck and Wurzinger, 1988).

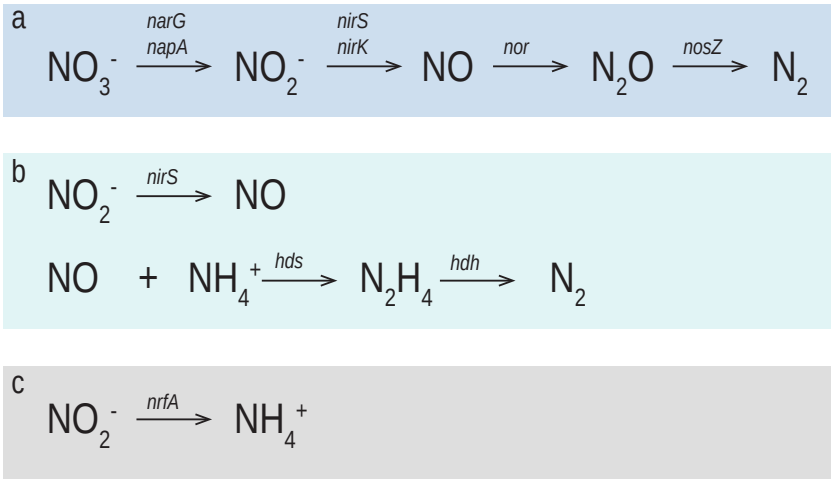

Figure 2. Microbially mediated anaerobic nitrogen transformations. a) Denitrification, b) anammox, c) dissimilatory nitrite reduction to ammonium, DNRA. Small text indicate genes encoding for the enzymes catalysing the reactions. In this thesis, *nirS*, *nirK*, *nosZ*, *hdh* and *nrfA* have been used as targets for quantification of abundances of functional bacterial communities.

The second step reduces nitrite to NO. The cytochrome-like and iron-dependent nitrite reductase cd_1NIR catalysing the reaction is encoded by *nirS* and the copper dependent Cu-NIR by *nirK*. Most organisms having a *nir* gene harbour only one them, but it has recently been shown that they can coexist in the same genome (Graf et al., 2014).

Nitric oxide is a cytotoxic molecule (Fang, 2004), so the third step in denitrification is, as the first step, a widespread trait. Microorganisms reduce NO

either for detoxification or for respiration and the group of enzymes responsible is diverse.

Finally, the N_2O may ultimately be reduced to N_2 by a copper dependent N_2O reductase, NOS. This microbial reaction is the only known sink of N_2O and about 60 % of the organisms having a *nir* gene also have *nosZ* (Graf et al., 2014). Two phylogenetically distinct clades of *nosZ* genes have evolved, *nosZ*I and *nosZ*II, and there are organisms that do not produce, but only reduce N_2O (Jones et al., 2013; Sanford et al., 2012). There is no gene for N_2O recuction found in fungi (Maeda et al., 2015).

Denitrification can also be autotroph, where hydrogen, iron or sulphur compounds serve as electron donors (Xing et al., 2018; Herrmann et al., 2017; Kumaraswamy et al., 2006).

Anammox

Anammox is a relatively recently discovered process (Mulder et al., 1995). Overall, ammonium is oxidised with nitrite as electron acceptor through a series of reactions that take place in the anammoxosome, a membrane bound structure in anammox bacteria. The end products of anammox are N_2 and water (Fig. 2b). One of the intermediates is the energy rich compound hydrazine formed from NO and ammonium in a complicated two-step reaction proposed to take place at different reactive sites within the same enzyme, hydrazine synthase, HZS, encoded by the gene *hds* (Dietl et al., 2015). Last, oxidation to N_2 is mediated by hydrazine dehydrogenase, HDH (Maalcke et al., 2016), also known by its former name hydrazine oxidoreductase, HZO (Schmid et al., 2008). The gene is named *hdh*, but consequently also known as *hzo*.

Anammox is only found in a limited number of bacteria, namely in five genera in the phylum Planctomycetes (Jetten, 2015). Ecologically, it has become evident that anammox plays an important role in the oceans and in oxygen minimum zones, where it contributes substantially to nitrogen removal (Dalsgaard et al., 2005). The anammox bacteria are autotrophs and growth is favoured by the absence of organic carbon sources (González-Cabaleiro et al., 2015; van de Graaf et al., 1996).

DNRA

DNRA is the reduction of nitrite to ammonium (Fig. 2c). The reaction is catalysed by the *nrfA* encoded formate-dependent cytochrome c nitrite reductase, in which the six-electron reduction of nitrite is performed without releasing any intermediate molecules (Einsle et al., 1999). Microorganisms can

use DNRA for growth, but the pathway might also be used for detoxification of nitrite or hydroxylamine, using other reductases (Kuypers et al., 2018).

The reduction can be performed by many microorganisms, including taxa within most bacterial lineages, but also among methane-oxidising archaea, and some diatoms and fungi (Kuypers et al., 2018; Kamp et al., 2015). Reducing conditions and high C/N ratios favour the process (Hardison et al., 2015; Kraft et al., 2014; Rütting et al., 2011), and it is found in anoxic environments such as wetlands, peatlands and sediments (Putz et al., 2018; Song et al., 2014; Davis et al., 2008).

2.2 Methods

Two methods for quantification of parameters important for understanding nitrogen turnover in anaerobic environments have been used extensively in this thesis.

Potential Denitrification Activity

A procedure for measuring the potential denitrification activity, PDA, based on acetylene inhibition was first described in 1976 (Yoshinari and Knowles, 1976). Since then, the method has been modified several times (e.g. Tiedje et al., 1989) and I have used the approach suggested by Pell et al. in 1996, recommending that the assay should be performed without chloramphenicol. Chloramphenicol not only inhibits the synthesis of new enzymes, but also affects the activity of already existing enzymes and the denitrification rates are thereby underestimated. Instead, the growth rate of the denitrifying community should be accounted for when calculating the denitrification rate.

The principle is to incubate a water slurry of the sample in a closed bottle under anoxic conditions with non-limiting concentrations of the substrates nitrate and carbon. The activity in the sample will hence be limited by the amount of the denitrifying enzymes in the sample. Acetylene is added to inhibit the activity of the nitrous oxide reductase, causing all N_2O produced to accumulate and preventing further reduction to N_2. The accumulation of N_2O is followed over time and the denitrification rate is calculated by regression of N_2O concentrations versus time. By omitting the carbon addition to the slurry, it can be determined if the activity is limited by carbon availability and by excluding acetylene, the net nitrous oxide production can be quantified.

There are a number of pitfalls when using PDA as a measure of denitrification in sediments. The N_2O produced is partly dissolved in the water phase of the slurry and to correctly account for that, the final slurry volume needs

to be determined in every single sample since the water content in sediment samples can vary substantially. The endemic denitrification capacity can be very low and long incubation times might be needed to produce detectable amounts of N_2O. Long incubation times can cause difficulties in interpreting the data. In the beginning of the incubation, the N_2O production rate is linear but with increasing time the microorganisms start growing and the relation between N_2O produced and time becomes exponential. When doing the regression, growth is accounted for, but it can be difficult to fit an exponential function to data with an extended lag phase. Low activity may also result in lower than air N_2O levels in the sampled gas, which needs to be recognized before performing the assay.

Quantitative PCR

Quantitative polymerase chain reaction (qPCR) has been used to study the abundance of specific microbial taxa and functional microbial communities in environmental samples the last 10-15 years (Graf et al., 2016; Wessén et al., 2011; Fierer et al., 2005). The basic principle for qPCR is to follow the exponential increase in the number of amplicons produced in the PCR reaction. This can be done by using DNA-binding fluorescent dyes and absolute quantification is done from standard curves, as in any analytical procedure. The many steps from DNA extract to number of gene copies per unit sample give numerous challenges along the way.

Quantitative PCR requires that the *Taq* DNA polymerase (*Taq*) is not hindered in its efficiency to amplify the DNA, otherwise the result will not be quantitative. Inhibition of the reaction can be due to sub-optimal performance of the *Taq* or because the target DNA is not accessible, both problems originating from inhibitory substances in the matrix present in the reaction mix. Inhibitors are often co-extracted with the DNA and different sample types have their typical inhibitors. Hence, it is crucial to confirm that the amplification in each single DNA extract is free from inhibition under the conditions used in the quantitative assay. This can be done in several ways, and the method used in this thesis is plasmid addition and comparing amplification in presence or absence of the sample.

In addition, it is often challenging to design primers for functional genes and one should bear in mind that only part of the target community is typically covered (Bonilla-Rosso et al., 2016). Further, due to variabilities in the primer target sites, degenerate primers are often used for functional genes. They contain a mix of primers to allow for the amplification of gene sequences that are similar but not identical. This contributes to an amplification efficiency below 95 %, which is often found when quantifying functional gene abundances in environmental samples.

3 Site Descriptions

The fieldwork connected to the papers in this thesis was performed at the mining company Loussavaara-Kiirunavaara Aktiebolag (LKAB) mine sites, in Malmberget (paper I) and in Kiruna (papers II and III).

3.1 Water flows and nitrogen transports at mine sites

During blasting operations, not all of the explosives loaded in the bore holes prepared detonate and the ammonium nitrate in the ANFO explosives is readily dissolved in the infiltrating groundwater. Undetonated explosives are also adsorbed to the waste rock and enters the process water via being washed out from waste rock deposits, percolating into the groundwater and again pumped up from the mine (Nilsson and Widerlund, 2017). Yet another route for the ammonium nitrate is via the processing plants, where it is washed out from the ore during the milling, separation and flotation processes.

The typical water transport ways at an underground mine are depicted in Figure 3. As sublevel caving is done below the ground water table, the surface and ground water leaching into the mine needs to be removed. The water is transported to high reservoirs via underground pump stations and is subsequently used in the processing plants. From the plants, the tailings slurry is pumped to a pond where the tailings are deposited. The water then flows into a clarification pond where it is retained before being recirculated to the reservoirs or being discharged to the receiving waters.

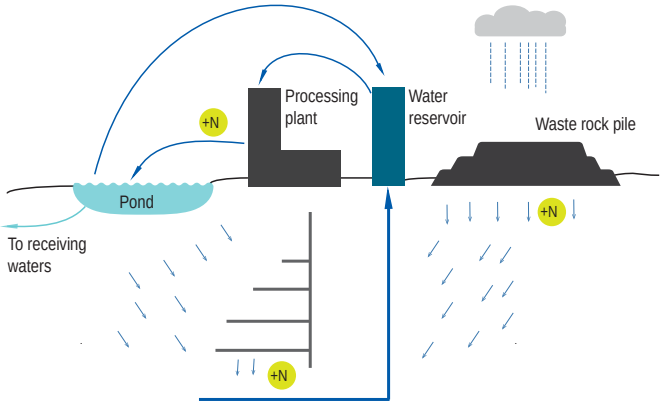

Figure 3. Water flows at a sublevel caving mine site. Points where nitrogen enters the flow paths are depicted with yellow circles.

3.2 The Kiruna and Malmberget mine sites

The LKAB mine sites are located in the north of Sweden. The region has a sub-arctic climate with mean annual temperatures below zero, Kiruna airport -1.7 °C and Malmberget -0.6 °C. At both locations, the temperature is above zero during the period May to September, but only June, July and August have mean temperatures above 10 °C (annual mean values 1961-1990, SMHI 2018). Both sites are surrounded by peatlands and deciduous forest dominated by birch and willows and the vegetation period is around 120 days (SLU 2006).

The two ponds at the Kiruna mine site (Fig. 4) have a total water volume of circa 3.0 Mm^3 and the retention time in the ponds is around 15 days. The water flows by gravity from the tailings pond into the clarification pond, after which most of it, 90 %, is recirculated to the reservoirs at the processing plants. The rest of the water leaves the pond by gravity and is discharged into the Mettä-Rakkurijoki water system, from there to the Kalix river and last into the Bothnian Bay. During the period 2015 to 2017, annually 9.4 Mm^3 water with a nitrate concentration of 27.3 mg L^{-1} was discharged from the clarification pond (mean values, LKAB environmental reports 2015-2017).

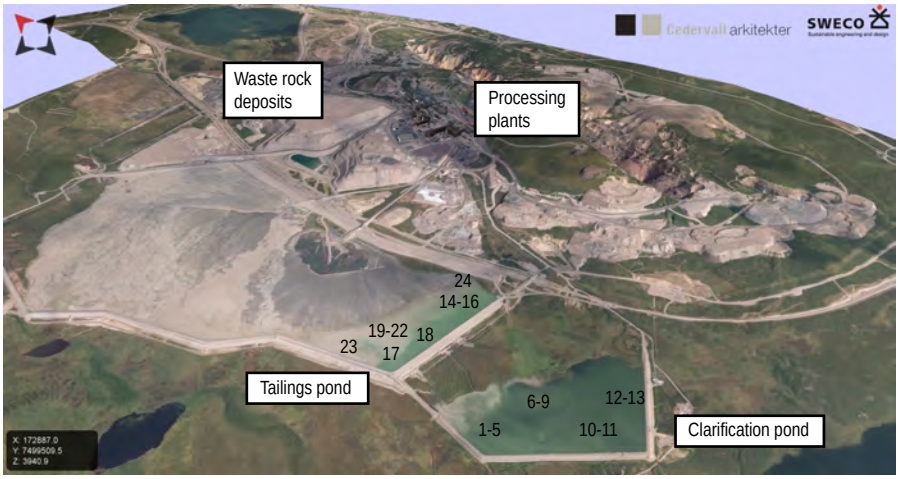

Figure 4. The LKAB mining area in Kiruna. The numbers in the ponds refer to the sampling points in paper III. Illustration: Matt Baida, Cedervall Arkitekter. Printed with permission.

At the Malmberget mine site the discharged water volume is smaller, 6.7 Mm^3 but the nitrate concentration at the discharge point is higher, 34.8 mg L^{-1} (mean values, LKAB environmental reports 2015-2017). The water from the ponds in the Malmberget mine area falls into the Lina river and finally also empties into the Bothnian Bay.

4 Bioreactors for nitrogen removal

Cellulose-based bioreactors for nitrogen removal from water can be constructed and operated at relatively low cost and in the last decade the technology has been well established (Christianson and Schipper, 2016). The main application is treating agricultural drainage rich in nitrate, whereas treatment of nitrogen - contaminated water from mining industry is so far less explored. A quick search in the Web of Science database (2018-07-16) for "denitrifying bioreactor" gave 244 results in the recent five years. Only five publications were left after refining the search using "mining" as a search term, and two of the five articles were directly connected to the same project(s) as in this thesis.

In principle, a cellulose-based bioreactor is an arrangement that allows contaminated water to flow through a porous organic material, the substrate. In the case of denitrifying reactors, the substrate releases organic carbon compounds that are used as electron donors when the nitrate is reduced to nitrogen gases via denitrification by denitrifying microorganisms. The substrate also serves as a surface for biofilm growth. The construction can be in the form of walls, layers or compartments/beds, and which design to choose depends on if the discharge is diffuse or concentrated, on hydrologic conditions and of the specific site constraints (Schipper et al., 2010b).

4.1 Substrate types and nitrate removal performance

The nitrate removal capacity of a bioreactor depends on its design, including the substrate used, as well as on other factors such as temperature, hydraulic retention time (HRT), influent nitrate concentrations and age.

Substrates

The most common material in field-scale reactors is woodchips; it is cheap, easily available, supports high permeability and has a high C:N ratio (Schipper

et al., 2010a). The slow release of carbon from woodchips has the advantage of giving the reactors long life lengths, 14-15 years have been reported (Long et al., 2011; Robertson et al., 2008) and nearly 40 years has been estimated (Warneke et al., 2011).

A recent meta-analysis including 57 field and laboratory scale woodchip reactors showed that nitrate removal rates in column experiments and bed type reactors are not significantly different from each other. Bed type reactors remove 4.7 g N m^{-3} day^{-1} and laboratory types 3.5 g N m^{-3} day^{-1} (Addy et al., 2016). The bed type reactors had significantly lower removal rates if the HRT were <6 hours or if the reactors were more than one year old. Nitrate removal rates were in the same range in a woodchip reactor built for treating mining water, where the average nitrogen removal was around 7 g N m^{-3} day^{-1} at a retention time of ca 2.4 days (Nordström and Herbert, 2018). Not surprising, the meta-analysis showed that temperature affected the reactor performance; the nitrogen removal rate increased more than 2 times per 10 °C increase (temperature range 3-20 °C). Other studies addressing temperature and HRT as factors determining woodchip reactor performance show similar results (Hoover et al., 2016; Nordström and Herbert, 2017).

Many other carbon rich substrates have been tested for their suitability as electron donors. The possibility of using waste products has been important when considering which materials to choose. Rice hulls, cotton, cardboard, seaweed, newspaper and numerous mixtures of these and others are substrates that have been tested (Fowdar et al., 2015; Della Rocca et al., 2006; Greenan et al., 2006; Ovez et al., 2006; Volokita et al., 1996). It seems like many cellulose-based substrates are suitable as electron donors in denitrifying bioreactors as long as the HRT can be adjusted.

Other factors influencing nitrate removal rates

The concentration of nitrate in the influent water is a rate-limiting factor (Addy et al., 2016). From a strict nitrogen removal point of view, it is of less importance; removal rates do not need to be high if the concentrations are low. However, if nitrate is fully depleted from the system other, non-wanted, biologically mediated chemical reactions can take place. The substrate carbon quality in terms of fibre composition also influences the removal rates (Schmidt and Clark, 2013) but there is no clear correlation between dissolved organic carbon (DOC) and removal (Fowdar et al., 2015). Denitrification can be enhanced by addition of external carbon, for example methanol. This is common practice in wastewater treatment plants but it is not typical for managing reactors in the field. Besides the practical and economical aspects, the fundamental idea is that these reactors need to function per se. To shorten the start-up phase of a

bioreactor, the substrate can be inoculated with denitrifying microorganisms. This can be particularly useful in laboratory experiments were reactors are fed with synthetic wastewater or groundwater (Zhao et al., 2018; Healy et al., 2012) but has also been used in field experiments (Nordström and Herbert, 2018, Mankiewicz-Boczek et al., 2017, paper I). In a reactor treating agricultural drainage this is not necessary; organisms capable of denitrification are ubiquitous in agricultural soils (Philippot et al., 2007) and will with time populate the substrate surfaces.

As described above, studies addressing nitrate removal in denitrifying bioreactors are diverse. The reported nitrate removal rates are based on woodchip reactors treating tile drainage from agricultural fields or on small-scale, 2 - 200 L, laboratory reactors packed with different substrates. Moreover, the reactors treat different types of contaminated water. The differences in both water types and other conditions make quantitative comparisons of nitrate removal efficiencies between studies difficult. Still, I conclude that the overall trends are more dependent on substrate type and HRT than on which type of water that infiltrates the bed.

4.2 Potential adverse effects

There is a potential risk of negative environmental effects with bioreactors. Environmentally harmful compounds can leave the system either as solutes in the effluent water or as gas emissions from the bed itself. It is important to have knowledge about the chemical and biological dynamics in the system to avoid swapping one environmental problem, nitrate, to another. Therefore, many studies in the area have focused on how to design and operate the reactors to mitigate the negative effects (Christianson and Schipper, 2016; Healy et al., 2012). In woodchip reactors, the potent greenhouse gas N_2O is mainly found dissolved in the water phase (Warneke et al., 2011) and conditions favouring N_2O production seem to be low temperature and low nitrate removal rates (Nordström and Herbert, 2018). Nevertheless, the overall N_2O emitted from woodchip reactors are considered minor (Healy et al., 2012). Nitrous oxide emissions from other substrates are found to be higher (Feyereisen et al., 2016; Warneke et al., 2011). Production of the greenhouse gas methane (CH_4) by methanogens can take place at reducing conditions and low levels of nitrate. At the start-up of a bioreactor, reducing conditions might occur due to high levels of DOC, but when CH_4 has been detected in woodchip reactors, it is in most cases transient (Nordström and Herbert, 2018) or in low concentrations (Warneke et al., 2011). With other substrate types, higher CH_4 emissions have been reported (Healy et al., 2012). The decrease in CH_4 emissions with time of

bioreactor operation can be explained either by the organic material being washed out or consumed. Washout of organic material is in itself an environmental risk since it can result in low oxygen levels in the receiving water bodies when the material is degraded. Initial high levels of carbon released from reactors with woodchips or a variety of other cellulose materials decline and stabilise (Grießmeier et al., 2017; Cameron and Schipper, 2010) although non-woodchip reactors seem to release more carbon than woodchip reactors (Feyereisen et al., 2016; Warneke et al., 2011). Control measures include regulation of HRT or the use of pre-treated (e.g. weathered or washed) woodchips (Christianson and Schipper, 2016; Schipper et al., 2010b). In addition to carbon and dissolved gases, nitrite and ammonium are non-wanted effluent solutes. Nitrite concentrations reported from woodchip reactors are in general low, or high only during the start-up period of a reactor (Nordström and Herbert 2018, paper I). Ammonium levels in both woodchip and other type of reactors also decrease after some time (Cameron and Schipper, 2010) and steady state ammonium levels of 1-6 mg N L^{-1} are reported (Healy et al., 2012). For comparison, the limit for fishing waters in Sweden is 1 mg L^{-1} as ammonium (SFS 2001:554).

4.3 Main removal pathways and microbial communities

Ideally, the nitrate will undergo full denitrification and be converted to N_2 while passing through the reactor. Denitrification being the nitrate removal pathway was suggested already in 1995 (Robertson and Cherry, 1995) and since then, several studies both at the field- and laboratory-scale, have verified that denitrification is the major nitrate removal process in woodchip reactors (Nordström and Herbert, 2018; Greenan et al., 2006; Schipper and Vojvodić-Vuković, 2000).

The competing process DNRA can occur if the C/N ratio is high. In woodchip reactors, Nordström and Herbert (2018) found that DNRA increases at temperatures below 5 °C and Greenan et al. (2006) reported that DNRA account for < 4 % of the total nitrate removal, although reports on DNRA in woodchip bioreactors are scarce. DNRA has been detected in reactors with other types of solid substrates, but only contributing to less than 15 % of the total nitrate reduction (Grau-Martínez et al., 2017; Fowdar et al., 2015).

In paper I, we reported low abundances of anammox, but in general, there are few reports on the pathway in denitrifying bioreactors. It is only mentioned as a conceivable but unlikely reaction as it is not likely to occur in systems with high availability of carbon (Schmidt and Clark, 2013; Warneke et al., 2011; Schipper et al., 2010a).

A handful of studies have investigated microbial community compositions in bioreactors using qPCR. It is difficult to generalize from these studies, as DNA extraction methods varies within and between studies, there are often high variability between replicate analyses and unlikely ratios between gene abundances are reported. Nevertheless, spatial patterns in functional gene abundances with depth and length of bioreactors have been reported (Andrus et al., 2014), as well as seasonal differences and recurring annual patterns in total bacterial communities (Porter et al., 2015). So far, the composition of the bacterial communities have not been extensively explored as DNA sequencing methods were only recently used to assign microbial communities in cellulose-based denitrifying reactors to taxonomic groups (Griessmeier and Gescher, 2018; Grießmeier et al., 2017). Hence, it is not known to which extent an established community originates from the inoculum or from the substrate itself. Hathaway et al. (2015) demonstrated that both total and denitrifying bacterial communities from several woodchip reactors were similar between the reactors and distinct from communities in nearby habitats, suggesting that the substrate rather than the inoculum drives the composition of the community.

4.4 The bioreactors studied in this thesis

In paper I, a pilot scale denitrifying bioreactor was constructed to treat nitrate-rich mine effluents from the LKAB mine site in Malmberget. It was constructed as a lined compartment bed-type of bioreactor with sawdust and gravel in a 27 m^3 container ("lined") and was placed directly on the ground. To establish steady flow, the reactor was operated for one season (May-October 2010). The actual experimental period with measurements of temperature, in- and outlet concentrations of reactive nitrogen species and alkalinity as well as the tracer test took place during the summer 2011. Substrate samples for DNA extraction were collected when the reactor was excavated in June 2012, directly after the winter period when it had been frozen.

The reactors in paper II were constructed in 0.5 L glass cylinders packed with three cellulose-based substrates, barley straw, the sedge *Carex rostrata* and pine woodchips. They were continuously fed with water from the clarification pond at the LKAB Kiruna mine site, at 10 °C for 10 months. The in- and outlet water chemistry was monitored regularly and material from the reactor bed was taken for DNA extraction and analyses of the microbial communities with monthly intervals.

Performance

In the Malmberget reactor, complete removal of the incoming nitrate was achieved (>95 %) during fail-free operation, corresponding to 5-10 g N m^{-3} (bed material) day^{-1} at a retention time of ca. 15 hours and temperatures between 15 and 21 °C (Fig. 2a, paper I). Although not directly comparable to a woodchip reactor, the nitrate removal capacity was within the range of what has been reported for other reactors (Addy et al., 2016). However, sufficient removal rates in the Malmberget reactor were only achieved after addition of external carbon in the form of acetate. Before the addition, only 14 – 47 % of the nitrate was removed. The carbon release rate from the sawdust was likely not high enough to support full removal of nitrate. Intuitively, the small grain size would offer more surface for biofilm growth for denitrifiers, but sawdust was not more effective when compared to woodchip (Schmidt and Clark, 2013). Instead, the proportion of organic material is important and the wood:sand ratio has been shown to correlate with nitrate removal (Schmidt and Clark, 2013). Thus, in the Malmberget reactor the ratio was probably too low as sawdust was mixed with three volumes of gravel, to allow for higher permeability.

The woodchip columns in paper II had a lower nitrate removal capacity, 1-2 g N m^{-3} day^{-1} after the start-up period of six months (Fig. 1, paper II). However, when expressing the rate per volume of substrate instead of volume of water, a rate of ~4 g N m^{-3} day^{-1} was obtained, which is very close to what has been found in lab-reactors in a meta-analysis (Addy et al., 2016). In accordance with other studies, the non-woodchip reactors in our study (Fig. 1, paper II) were significantly more efficient than woodchip reactors in removing nitrate (Warneke et al., 2011; Schipper et al., 2010b; Greenan et al., 2006).

Apart from removing nitrate, the woodchip reactors also produced nitrite, especially in the beginning of the experiment, whereas the straw and sedge reactors had outlet nitrite concentrations close to the inlet concentrations (Fig. S1a, paper II). For ammonium, there was no significant change between in- and outlet concentrations at any of the time points evaluated in the woodchip reactors, but accumulation of ammonium was observed in the straw and sedge reactors (Fig. S1b, paper II). Initial flushes of the ions could be a simple washout-from-the-substrate effect, differing between substrates but nitrite and ammonium could also have been produced by the microorganisms present in the substrate. To better understand why the substrates performed differently from each other, we investigated the microbial communities performing the different reaction pathways underlying the contrasting patterns and nitrate removal rates.

Functional groups involved in nitrogen transformations

In both paper I and paper II, we investigated the abundance of the denitrifying and anammox communities using qPCR. To my best knowledge, there are no studies specifically addressing these communities in cellulose-based bioreactors treating mine effluents. Existing reports from mine water treatments are either from other types of reactors or only investigate the total bacterial communities (Papirio et al., 2014; Karkman et al., 2011).

In comparison to denitrification, anammox did not seem to be an important process based on the qPCR data. Genes associated with anammox were found in the Malmberget reactor, but the abundances were very low, suggesting that this is not a major nitrate removal pathway (paper I). Anammox could not be detected in the reactors in paper II. However, since different primer pairs were used in the two studies, the results are not comparable. A taxa-specific part of the 16S rRNA gene was used in paper I and in paper II the qPCR target was the functional gene *hdh* (*hzo*).

The abundances of the denitrification genes differed in the different substrates in the paper II bioreactors (Fig. 4c-f, paper II). With exception of the *nosZ*I gene, all gene abundances were lower in the woodchip substrate compared to straw and sedge, which could be a rationale behind the lower nitrate removal rates in the woodchip reactors. In contrast to in many other habitats (Jones et al. 2013), the ratios between *nosZ*II and *nosZ*I abundances were below 1 for the substrates and reactor types studied here. The *nosZ*II gene dominate among N_2O-reducing bacteria that do not denitrify (Graf et al., 2014, Jones et al., 2013). This suggests that *nosZ*I, most often found in denitrifiers, are favoured under denitrifying conditions and could hence be an explanation for the high abundance of *nosZ*I relative to *nosZ*II found in our bioreactors (papers I and II). The ratio between *nos* and *nir* genes gives information about the potential for reduction of N_2O produced in a system. All substrate types in the lab-scale reactors had a lower ratio, i.e. higher risk, of emitting N_2O compared to the pilot-scale reactor. The lower temperature in the lab-scale reactors could be an explanation as low temperature increase the risk of N_2O-production (Nordström and Herbert, 2018).

In paper II we also quantified the abundances of the functional marker gene *nrfA*, indicating DNRA. In accordance with the accumulation of ammonium in the straw and sedge reactors, these two substrates both displayed higher genetic potential for the production of ammonium by DNRA (Fig. 4b, paper II).

Water may flow in preferential paths through the reactor bed, leading to that the entire volume of the bed might not be efficiently used. We investigated if there were spatial variation in the abundances of functional groups in the reactor material, indicating preferential flow paths. No spatial variation was detected in

the lab-reactors in paper II, but variations in all three dimensions were found in the pilot-scale Malmberget reactor in paper I. The closed glass cylinders in the laboratory experiment probably provided a more homogenous environment and were operated during a shorter time period, and thereby had less fluctuating communities. These reactors were nearly always fully water saturated, operated at constant temperature, receiving the same water and with no depletion of nitrate, while the pilot-scale reactor was operated at ambient conditions, allowing for differences in oxygen availability and fluctuations in temperature. Moreover, the sawdust in the pilot-scale reactor could likely move due to flowing water and thereby preferential flow paths were developed (paper I). In contrast to our study in the Malmberget reactor, Andrus et al. (2014) did not find spatial variations in the denitrifying community, but this conclusion was based only on *nosZ* gene fingerprints. In the Malmberget reactor, the *nosZ* community varied less than the *nirS* and *nirK* communities in the longitudinal transects. Divergent colonization patterns between the *nosZ*I and *nosZ*II communities, spatially in the pilot-reactor and substrate dependent in the lab-reactors, indicate that the organisms belonging to the two clades have various responses to the environmental conditions.

Microbial community composition and diversity

The community composition and diversity in the lab-reactors were investigated over the course of the experiment by sequencing part of the 16S rRNA gene (paper II) and we showed that the microbial community that established in a reactor was depending on substrate type. During the first five-six months, the alpha-diversities in all substrates increased after which they remained stable until the end of the experiment. The woodchip reactors had the lowest alpha-diversities while the diversities in the straw and sedge reactors were higher (Fig. 5).

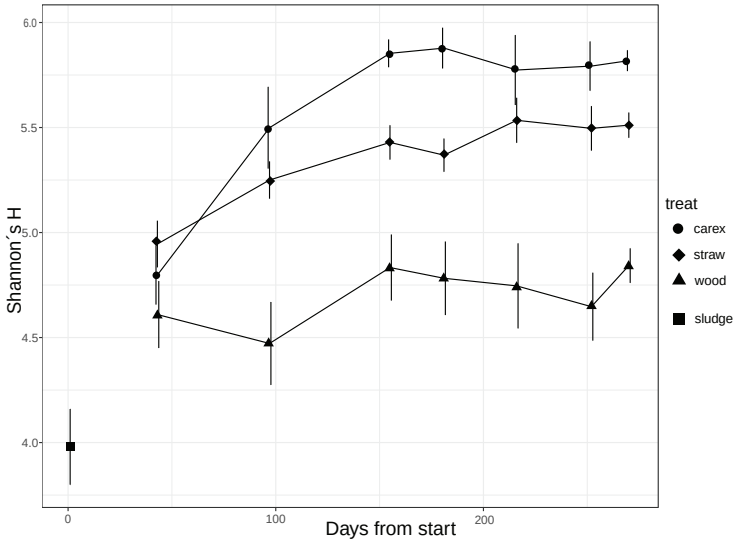

Figure 5. Shannon´s diversity index in the sludge used for inoculating the reactors and in the different reactor types during the experimental period (mean, n=3).

The approximate time point where the plateau levels were established coincided with the patterns observed for the gene abundances and nitrate removal. It was most clear in the woodchip reactors, where the abundances of all genes quantified, with the exception of *nosZ*I, remained unchanged, as did the nitrate removal rate (Figs. 1 and 4, paper II). The three substrates had their own distinct community patterns, developed over time, as was shown by a non-metric multidimensional scaling (NMDS) analysis (Fig. 2, paper II). The microbial community composition in the inoculum sludge was also unique.

5 Nitrogen removal in pond sediments

Tailings and clarification impoundments at mine sites give opportunities for nitrogen removal. Their large areas and long water retention times are important for the several ways in which nitrogen can be transformed. Nitrogen uptake in biomass is obvious if the pond is vegetated, but even if macrophytes are sparse or missing, nitrogen can be assimilated by phytoplankton. Further, nitrogen can be retained by permanent burial in the sediments. Nitrogen can also be permanently removed by microorganisms present in the sediment that can reduce nitrate via denitrification or anammox. It has been suggested that denitrification contributes the most to nitrogen removal in mine impacted waters (Chlot et al., 2011).

Denitrification takes place in the sediments of many water bodies. In wetlands, nitrate is often rate limiting for denitrification (Kjellin et al., 2007). However, availability of labile carbon can be the rate-limiting factor in lakes and riparian zones with groundwater inflow (Trauth et al., 2018; Stoliker et al., 2016) but also in mine ponds (Nilsson and Widerlund, 2018). The origin of organic carbon in sediments can be settled phytoplankton and detritus from plants (Hellemann et al., 2017; Bastviken et al., 2005) and a previous study at the Kiruna mine site suggests that a significant proportion of the organic matter in the pond sediments come from phytoplankton (Ecke et al., 2013). In the absence of organic carbon, autotrophic denitrification utilizing other electron donors can take place. This might be the case in ponds at mining sites, since the content of organic carbon is low and sulphur compounds and metal ions are often present.

5.1 Findings in the Kiruna mine site ponds

To better understand the nitrogen removal potential in the Kiruna mine site ponds, we measured chemical, biochemical and biological parameters in the water and sediments (paper III). We sampled sediment and water from the

tailings and clarification ponds (Fig. 4). Chemically, the ponds differ from each other in both water and sediment parameters, with the tailings pond having higher levels of the anions sulphate, nitrite, nitrate and ammonium as well as of total phosphorous in the water (Supplementary Table 1, paper III). A principal components analysis clearly distinguishes the differences within and between the ponds (supplementary Fig. 1, paper III). The clarification pond had higher levels of total organic carbon in the water, reflected also in the sediment organic and total carbon content. Despite the differences in carbon content, no difference in endogenous denitrification rate (i.e. without adding carbon to the assay) was found between the two ponds but as expected, the denitrification rates after adding carbon increased significantly in both ponds (Fig. 6).

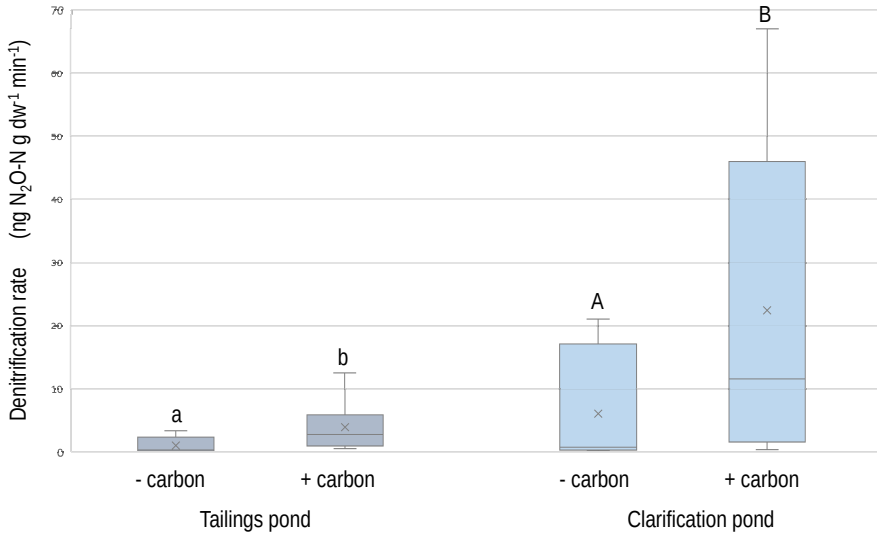

Figure 6. Potential denitrification activity in the tailings and clarification ponds at the LKAB mine site in Kiruna with and without addition of carbon. x within boxes denote the mean. Different letters above the boxes indicate significant differences within each pond ($p<0.05$). Tailings pond n=8, clarification pond n=10.

The heterogeneities in the sediments´ chemical compositions and in denitrification rates were also reflected in the bacterial community compositions. Similar to the ordination based on the chemical parameters, the NMDS displayed the sampling clusters in the clarification pond (Fig. 7). In addition, the samples from the deeper area in the tailings pond grouped together. The alpha-diversity did not differ between the ponds but the community composition did (Fig. 5a-b, paper III). Bacteria in the orders Flavobacteriales and Pseudomonadales dominated both ponds. In the tailings pond, order

Oscillatoriales, genus *Phormidium* was present, originating from one single sample, a shallow spot where biofilm was clearly visible on the surface of the sediment. Only the tailings pond had representatives for microorganisms capable of autotroph denitrification, genera *Rhodobacter* and *Sulfuritalea*, logic from the lower organic carbon content in sediment and water.

5.2 Carbon additions to pond sediments

Since the denitrification rates in both ponds were limited by available organic carbon, we performed a microcosm experiment, where sediment from the clarification pond was amended with carbon to see if long-term treatment would increase denitrification (paper III). We chose algae and a cellulose type of carbon because of their photosynthetic origin. Supporting algal growth in the ponds could potentially be a way of supporting denitrification in the ponds. Today, phytoplankton growth in the ponds is limited by phosphorous (Chlot et al., 2013) so altering the P levels would be needed to increase growth.

Both algae and acetate addition resulted in complete removal of nitrate (i.e. nitrate+nitrite) from the water after approximately three weeks (Fig. 3a, paper III). After the treatment period, the potential denitrification rates in the sediments had increased in the algae treatment and in the positive control with acetate. The negative control and the hydroxyethylcellulose (HEC) treatment resulted in lower potential denitrification rates, likely because the organic carbon originally present in the sediment had been depleted (Fig. 2, paper III). In the HEC treatment, the total organic carbon in the overlaying water was increasing and reached a constant high level, indicating that the compound was not degraded and could not be used for denitrification (Fig. 3c, paper III). Although additions of detritus promote denitrification rates (Bastviken et al., 2005), HEC was obviously not a good choice to represent decaying plant material.

The increase in denitrification rates in the algae and acetate was not reflected by the abundances of *nirS* and *nirK* genes as they did not increase (Supplementary Table 4, paper III). A similar result was found in boreal lakes by Saarenheimo et al. (2015), other factors must have determined the denitrification rates. The quality of the added carbon most probably played a role; acetate is processed directly in the cell whereas the more complex algae carbon needed degradation before it could be used as electron donor. The metabolic pathways needed to exploit the different carbon resources is one factor determining which specific taxa were enriched for in the four treatments. However, the qPCR data helps in interpreting why the production of N_2O was lowest in the acetate treatment. In this treatment, the ratio between *nos* and *nir* genes was about three times higher than in the other treatments, showing a higher

potential for consumption of produced N_2O (Fig. 4, paper III). The qPCR data also pointed to that anammox was minor in relation to denitrification and that DNRA could be an important nitrate reduction pathway in the algae treatment (Fig. 4, paper III). The latter coincided with the observed ammonium dynamics

In line with the other parameters, the sediment treated with HEC and sediment in the water control did not differ from each other regarding community composition (Fig. 7) or alpha-diversity. Treatments resulting in higher denitrification rates had lower alpha-diversities and community compositions differing from the other treatments. Nearly half of the sequences in the algae treatment represented the order Bacteroidales, which is not surprising since many bacteria belonging to this taxon are known to degrade high molecular weight organic compounds. The acetate treatment had high numbers of Desulfobacterales indicating sulphur reduction, a process that could be detected by sulphide odour in the later half of the experiment.

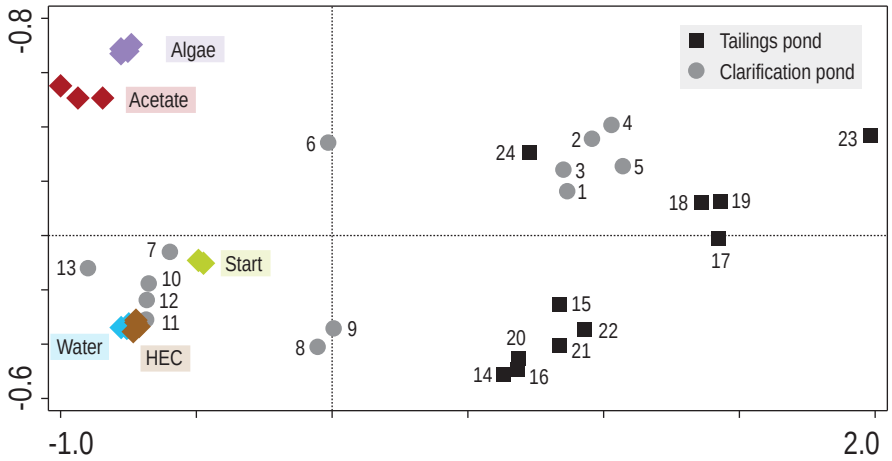

Figure 7. Non-metric multidimensional scaling of the bacterial communities in the sediment samples from the ponds and from the microcosm experiment. Pond samples numbered according to Fig. 4 and colour indicate sample origin. Microcosm treatments and the original sediment sample used in the microcosm experiment are shown as coloured diamonds. Stress value=0.040.

Based on the microcosm experiment in paper III, we conclude that although algae improved denitrification, negative environmental impacts were detected. We found that this treatment caused substantial CH_4 and N_2O production, and the ammonium levels in the water were orders of magnitudes higher than in the other treatments. The amount of carbon added in the lab-experiment was four times the stoichiometric demand for denitrification and this could of course be more balanced to avoid adding excess carbon leading to unwanted processes like DNRA or methanogenesis.

6 Summary and concluding remarks

The aim of this thesis was to investigate and identify factors that underpin microbial nitrogen removal from mining process water.

In paper I we reported on a field bioreactor treating mining discharge water and showed that denitrification was the main nitrate removal pathway. However, addition of acetate was needed to achieve high efficiency. After addition of acetate, the nitrate removal capacity was in the range of woodchip reactors treating agricultural drainage, 5-10 g N m^{-3} day^{-1}. There were also strong indications that preferential flows developed in the reactor. If so, only a part of the total volume of the denitrifying bed contributed to the removal. The change in hydraulic conditions might have been a result of clogging in the reactor caused by the fine grain size of the sawdust and by biofilm growth on the surfaces of the substrate. Spatial distribution patterns were found for the denitrifying bacterial community, with higher abundances in the deeper, more water saturated levels and distinct patterns for the *nirS* and *nirK* genes along the flow paths through the reactor. For future reactors, the problems with the hydraulic conditions might be overcome with improving the design of the in- and outlet pipes and woodchips would potentially be a better substrate choice compared to the sawdust/gravel mixture used in the pilot-reactor in paper I.

In paper II, we investigated how nitrate removal capacities and microbial community compositions in lab-scale reactors containing woodchips, barley straw or the sedge *Carex rostrate* developed over time. All reactor types reduced the nitrate by denitrification without need for external carbon additions. Nevertheless, nitrate removal rates differed between the substrate types. The woodchip reactors had lower removal rates compared to straw and *Carex*, likely explained by the lower abundances of denitrifiers present in these reactors. Unique bacterial communities established in each of the three substrates, although all reactors were inoculated with the same sludge and fed with the same water. This clearly points to the importance of the substrate properties, both in terms of endogenous microbial communities and of chemical composition, and

potentially also physical properties of the substrate. The reason for testing sedge as bioreactor substrate was based on a previous finding in the miNing project; *Carex rostrata* is a very efficient plant for nitrogen uptake in biomass and a good candidate plant for constructed wetlands for nitrogen removal at the Kiruna mine site. Harvesting the sedge at the end of the growth season and using it as a bioreactor substrate would not only remove nitrogen by assimilation, but also supply the energy needed for denitrification in the reactor. Mixing sedge into the woodchip bed may be a way of increasing the efficiency in denitrifying reactors. Approaches with mixed reactor material have been tested, but not in field scale. It remains to be tested and focus must be on potential adverse effects and life length of such mixed-substrate bioreactors.

In paper III, we found that the denitrification rates in the pond sediments at the Kiruna mine site are limited by organic carbon availability. We also showed that the two ponds differed in their community compositions. Further, we found that treatment with carbon in the form of algae enhanced denitrification. Even though the denitrification rate increased with algae treatment, the abundance of *nir* genes did not, again pointing to the specific composition of the microbial community as a determining factor for nitrate removal. Moreover, negative effects in the form of release of ammonium and CH_4 were detected in the algae treatment. These are some of the challenges that need to be considered when attempting to increase the denitrification rates in the pond sediments.

References

Addy, K., Gold, A. J., Christianson, L. E., David, M. B., Schipper, L. A. & Ratigan, N. A. 2016. Denitrifying Bioreactors for Nitrate Removal: A Meta-Analysis. *Journal of Environmental Quality,* 45(3)**,** pp 873-881.

Andrus, J. M., Porter, M. D., Rodriguez, L. F., Kuehlhorn, T., Cooke, R. A., Zhang, Y., Kent, A. D. & Zilles, J. L. 2014. Spatial variation in the bacterial and denitrifying bacterial community in a biofilter treating subsurface agricultural drainage. *Microbial Ecology,* 67(2)**,** pp 265-272.

Bastviken, S. K., Eriksson, P. G., Premrov, A. & Tonderski, K. 2005. Potential denitrification in wetland sediments with different plant species detritus. *Ecological Engineering,* 25(2)**,** pp 183-190.

Bergström, A. K., Faithfull, C., Karlsson, D. & Karlsson, J. 2013. Nitrogen deposition and warming - effects on phytoplankton nutrient limitation in subarctic lakes. *Global Change Biology,* 19(8)**,** pp 2557-2568.

Bonilla-Rosso, G., Wittorf, L., Jones, C. M. & Hallin, S. 2016. Design and evaluation of primers targeting genes encoding NO-forming nitrite reductases: implications for ecological inference of denitrifying communities. *Scientific Reports,* 6:39208.

Camargo, J. A. & Alonso, Á. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. *Environment International,* 32(6)**,** pp 831-849.

Cameron, S. G. & Schipper, L. A. 2010. Nitrate removal and hydraulic performance of organic carbon for use in denitrification beds. *Ecological Engineering,* 36(11)**,** pp 1588-1595.

Chen, J. & Strous, M. 2013. Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1827(2)**,** pp 136-144.

Chlot, S., Widerlund, A., Husson, E., Ohlander, B. & Ecke, F. 2013. Effects on nutrient regime in two recipients of nitrogen-rich mine effluents in northern Sweden. *Applied Geochemistry,* 31, pp 12-24.

Chlot, S., Widerlund, A., Siergieiev, D., Ecke, F., Husson, E. & Ohlander, B. 2011. Modelling nitrogen transformations in waters receiving mine effluents. *Science of the Total Environment,* 409(21)**,** pp 4585-4595.

Christianson, L. E. & Schipper, L. A. 2016. Moving Denitrifying Bioreactors beyond Proof of Concept: Introduction to the Special Section. *Journal of Environmental Quality,* 45(3)**,** pp 757-761.

Dalsgaard, T., Thamdrup, B. & Canfield, D. E. 2005. Anaerobic ammonium oxidation (anammox) in the marine environment. *Research in Microbiology,* 156(4)**,** pp 457-464.

Davis, J. H., Griffith, S. M., Horwath, W. R., Steiner, J. J. & Myrold, D. D. 2008. Denitrification and nitrate consumption in an herbaceous riparian area and perennial ryegrass seed cropping system. *Soil Science Society of America Journal,* 72(5)**,** pp 1299-1310.

Della Rocca, C., Belgiorno, V. & Meric, S. 2006. An heterotrophic/autotrophic denitrification (HAD) approach for nitrate removal from drinking water. *Process Biochemistry,* 41(5)**,** pp 1022-1028.

Dietl, A., Ferousi, C., Maalcke, W. J., Menzel, A., de Vries, S., Keltjens, J. T., Jetten, M. S. M., Kartal, B. & Barends, T. R. M. 2015. The inner workings of the hydrazine synthase multiprotein complex. *Nature,* 527(7578)**,** pp 394-404.

Ecke, F., Hallin, S., Hellman, M., Herbert, R., Widerlund, A. 2013. Feasibility Study: Minimizing nitrogen releases for LKAB. Project report.

Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G. P., Bartunik, H. D., Huber, R. & Kroneck, P. M. H. 1999. Structure of cytochrome c nitrite reductase. *Nature,* 400(6743)**,** pp 476-480.

EU Commission, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 Octorber 2000 establishing a framework for community aciton in the field of water policy. *Official Journal of the European Communities.*

Fang, F. C. 2004. Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies. *Nature Reviews Microbiology,* 2(10)**,** pp 820-832.

Feyereisen, G. W., Moorman, T. B., Christianson, L. E., Venterea, R. T., Coulter, J. A. & Tschirner, U. W. 2016. Performance of Agricultural Residue Media in Laboratory Denitrifying Bioreactors at Low Temperatures. *Journal of Environmental Quality,* 45(3)**,** pp 779-787.

Fierer, N., Jackson, J. A., Vilgalys, R. & Jackson, R. B. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Applied and Environmental Microbiology,* 71(7)**,** pp 4117-4120.

Forsyth, B., Cameron, A. & Miller, S. 1995. Explosives and water quality. *Proceedings of Sudbury '95 Mining and the Environment,* pp 795-803.

Fowdar, H. S., Hatt, B. E., Breen, P., Cook, P. L. M. & Deletic, A. 2015. Evaluation of sustainable electron donors for nitrate removal in different water media. *Water Research,* 85, pp 487-496.

González-Cabaleiro, R., Ofiţeru, I. D., Lema, J. M. & Rodríguez, J. 2015. Microbial catabolic activities are naturally selected by metabolic energy harvest rate. *The ISME Journal,* 9, 2630-2641.

Graf, D. R. H., Jones, C. M. & Hallin, S. 2014. Intergenomic Comparisons Highlight Modularity of the Denitrification Pathway and Underpin the Importance of Community Structure for N2O Emissions. *Plos One,* 9(12)**,** e114118.

Graf, D. R. H., Zhao, M., Jones, C. M. & Hallin, S. 2016. Soil type overrides plant effect on genetic and enzymatic N_2O production potential in arable soils. *Soil Biology & Biochemistry,* 100, pp 125-128.

Grau-Martínez, A., Torrentó, C., Carrey, R., Rodríguez-Escales, P., Domènech, C., Ghiglieri, G., Soler, A. & Otero, N. 2017. Feasibility of two low-cost organic substrates for inducing denitrification in artificial recharge ponds: Batch and flow-through experiments. *Journal of Contaminant Hydrology,* 198, pp 48-58.

Greenan, C. M., Moorman, T. B., Kaspar, T. C., Parkin, T. B. & Jaynes, D. B. 2006. Comparing carbon substrates for denitrification of subsurface drainage water. *Journal of Environmental Quality,* 35(3)**,** pp 824-829.

Grießmeier, V., Bremges, A., McHardy, A. C. & Gescher, J. 2017. Investigation of different nitrogen reduction routes and their key microbial players in wood chip-driven denitrification beds. *Scientific Reports,* 7:17028.

Griessmeier, V. & Gescher, J. 2018. Influence of the Potential Carbon Sources for Field Denitrification Beds on Their Microbial Diversity and the Fate of Carbon and Nitrate. *Frontiers in Microbiology,* 9:1313.

Hardison, A. K., Algar, C. K., Giblin, A. E. & Rich, J. J. 2015. Influence of organic carbon and nitrate loading on partitioning between dissimilatory nitrate reduction to ammonium (DNRA) and N-2 production. *Geochimica Et Cosmochimica Acta,* 164, pp 146-160.

Hathaway, S. K., Porter, M. D., Rodríguez, L. F., Kent, A. D. & Zilles, J. L. 2015. Impact of the contemporary environment on denitrifying bacterial communities. *Ecological Engineering,* 82, pp 469-473.

Hayakawa, A., Hatakeyama, M., Asano, R., Ishikawa, Y. & Hidaka, S. 2013. Nitrate reduction coupled with pyrite oxidation in the surface sediments of a sulfide-rich ecosystem. *Journal of Geophysical Research-Biogeosciences,* 118(2)**,** pp 639-649.

Healy, M. G., Ibrahim, T. G., Lanigan, G. J., Serrenho, A. J. & Fenton, O. 2012. Nitrate removal rate, efficiency and pollution swapping potential of different organic carbon media in laboratory denitrification bioreactors. *Ecological Engineering,* 40, pp 198-209.

Heiss, A. G. & Oeggl, K. 2008. Analysis of the fuel wood used in Late Bronze Age and Early Iron Age copper mining sites of the Schwaz and Brixlegg area (Tyrol, Austria). *Vegetation History and Archaeobotany,* 17(2)**,** pp 211-221.

Hellemann, D., Tallberg, P., Bartl, I., Voss, M. & Hietanen, S. 2017. Denitrification in an oligotrophic estuary: a delayed sink for riverine nitrate. *Marine Ecology Progress Series,* 583, pp 63-80.

Herrmann, M., Opitz, S., Harzer, R., Totsche, K. U. & Kusel, K. 2017. Attached and Suspended Denitrifier Communities in Pristine Limestone Aquifers Harbor High Fractions of Potential Autotrophs Oxidizing Reduced Iron and Sulfur Compounds. *Microbial Ecology,* 74(2)**,** pp 264-277.

Hoover, N. L., Bhandari, A., Soupir, M. L. & Moorman, T. B. 2016. Woodchip Denitrification Bioreactors: Impact of Temperature and Hydraulic Retention Time on Nitrate Removal. *Journal of Environmental Quality,* 45(3)**,** pp 803-812.

Jermakka, J., Wendling, L., Sohlberg, E., Heinonen, H. & Vikman, M. 2015. Potential Technologies for the Removal and Recovery of Nitrogen Compounds From Mine and Quarry Waters in Subarctic Conditions. *Critical Reviews in Environmental Science and Technology,* 45(7)**,** pp 703-748.

Jetten, M., Op den Kamp, H., Kuenen, J., Strous, M. 2015. "Candidatus Brocadiaceae" fam. nov. *In: Bergey's Manual of Systematics of Archaea and Bacteria.* John Wiley & Sons, Inc.

Jones, C. M., Graf, D. R. H., Bru, D., Philippot, L. & Hallin, S. 2013. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME Journal,* 7(2)**,** pp 417-426.

Kamp, A., Høgslund, S., Risgaard-Petersen, N. & Stief, P. 2015. Nitrate Storage and Dissimilatory Nitrate Reduction by Eukaryotic Microbes. *Frontiers in Microbiology,* 6:1492.

Karkman, A., Mattila, K., Tamminen, M. & Virta, M. 2011. Cold Temperature Decreases Bacterial Species Richness in Nitrogen-Removing Bioreactors Treating Inorganic Mine Waters. *Biotechnology and Bioengineering,* 108(12)**,** pp 2876-2883.

Kjellin, J., Hallin, S. & Worman, A. 2007. Spatial variations in denitrification activity in wetland sediments explained by hydrology and denitrifying community structure. *Water Research,* 41(20)**,** pp 4710-4720.

Kraft, B., Tegetmeyer, H. E., Sharma, R., Klotz, M. G., Ferdelman, T. G., Hettich, R. L., Geelhoed, J. S. & Strous, M. 2014. The environmental controls that govern the end product of bacterial nitrate respiration. *Science,* 345(6197)**,** pp 676-679.

Kumaraswamy, R., Sjollema, K., Kuenen, G., van Loosdrecht, M. & Muyzer, G. 2006. Nitrate-dependent Fe(II)EDTA (2-) oxidation by Paracoccus ferrooxidans sp nov., isolated from a denitrifying bioreactor. *Systematic and Applied Microbiology,* 29(4)**,** pp 276-286.

Kuypers, M. M. M., Marchant, H. K. & Kartal, B. 2018. The microbial nitrogen-cycling network. *Nature Reviews Microbiology,* 16(5)**,** pp 263-276.

Long, L. M., Schipper, L. A. & Bruesewitz, D. A. 2011. Long-term nitrate removal in a denitrification wall. *Agriculture Ecosystems & Environment,* 140(3-4)**,** pp 514-520.

Maalcke, W. J., Reimann, J., de Vries, S., Butt, J. N., Dietl, A., Kip, N., Mersdorf, U., Barends, T. R. M., Jetten, M. S. M., Keltjens, J. T. & Kartal, B. 2016. Characterization of Anammox Hydrazine Dehydrogenase, a Key N-2-producing Enzyme in the Global Nitrogen Cycle. *Journal of Biological Chemistry,* 291(33)**,** pp 17077-17092.

Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C. & Philippot, L. 2015. N2O production, a widespread trait in fungi. *Scientific Reports,* 5:9697.

Mankiewicz-Boczek, J., Bednarek, A., Gągała-Borowska, I., Serwecińska, L., Zaborowski, A., Kolate, E., Pawełczyk, J., Żaczek, A., Dziadek, J. & Zalewski, M. 2017. The removal of nitrogen compounds from farming wastewater – The effect of different carbon substrates and different microbial activators. *Ecological Engineering,* 105, pp 341-354.

Mulder, A., Vandegraaf, A. A., Robertson, L. A. & Kuenen, J. G. 1995. Anaerobic Ammonium Oxidation Discovered in a Denitrifying Fluidized-Bed Reactor. *FEMS Microbiology Ecology,* 16(3)**,** pp 177-183.

Nilsson, L. & Widerlund, A. 2017. Tracing nitrogen cycling in mining waters using stable nitrogen isotope analysis. *Applied Geochemistry,* 84, pp 41-51.

Nilsson, L. & Widerlund, A. 2018. Modelling tool for predicting and simulating nitrogen concentrations in cold-climate mining ponds. *Ecological Modelling,* 380, pp 40-52.

Nordström, A. & Herbert, R. B. 2017. Denitrification in a low-temperature bioreactor system at two different hydraulic residence times: laboratory column studies. *Environmental Technology,* 38(11)**,** pp 1362-1375.

Nordström, A. & Herbert, R. B. 2018. Determination of major biogeochemical processes in a denitrifying woodchip bioreactor for treating mine drainage. *Ecological Engineering,* 110, pp 54-66.

Ovez, B., Mergaert, J. & Saglam, M. 2006. Biological denitrification in drinking water treatment using the seaweed Gracilaria verrucosa as carbon source and biofilm carrier. *Water Environment Research,* 78(4)**,** pp 430-434.

Papirio, S., Ylinen, A., Zou, G., Peltola, M., Esposito, G. & Puhakka, J. A. 2014. Fluidized-bed denitrification for mine waters. Part I: low pH and temperature operation. *Biodegradation,* 25(3)**,** pp 425-435.

Pell, M., Stenberg, B., Stenstrom, J. & Torstensson, L. 1996. Potential denitrification activity assay in soil - With or without chloramphenicol? *Soil Biology & Biochemistry,* 28(3)**,** pp 393-398.

Philippot, L., Hallin, S. & Schloter, M. 2007. Ecology of denitrifying prokaryotes in agricultural soil. *In:* Sparks, D. L. (ed.) *Advances in Agronomy, Vol 96.*

Porter, M. D., Andrus, J. M., Bartolerio, N. A., Rodriguez, L. F., Zhang, Y., Zilles, J. L. & Kent, A. D. 2015. Seasonal Patterns in Microbial Community Composition in Denitrifying Bioreactors Treating Subsurface Agricultural Drainage. *Microbial Ecology,* 70(3)**,** pp 710-723.

Putz, M., Schleusner, P., Rutting, T. & Hallin, S. 2018. Relative abundance of denitrifying and DNRA bacteria and their activity determine nitrogen retention or loss in agricultural soil. *Soil Biology & Biochemistry,* 123, pp 97-104.

Risgaard-Petersen, N., Langezaal, A. M., Ingvardsen, S., Schmid, M. C., Jetten, M. S. M., Op den Camp, H. J. M., Derksen, J. W. M., Piña-Ochoa, E., Eriksson, S. P., Peter Nielsen, L., Peter Revsbech, N., Cedhagen, T. & van der Zwaan, G. J. 2006. Evidence for complete denitrification in a benthic foraminifer. *Nature,* 443, pp 93-96.

Robertson, W. D. & Cherry, J. A. 1995. In-Situ Denitrification of Septic-System Nitrate Using Reactive Porous-Media Barriers - Field Trials *Ground Water,* 33(1)**,** pp 99-111.

Robertson, W. D., Vogan, J. L. & Lombardo, P. S. 2008. Nitrate removal rates in a 15-year-old permeable reactive barrier treating septic system nitrate. *Ground Water Monitoring and Remediation,* 28(3)**,** pp 65-72.

Rütting, T., Boeckx, P., Müller, C. & Klemedtsson, L. 2011. Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. *Biogeosciences,* 8(7)**,** pp 1779-1791.

Saarenheimo, J., Tiirola, M. A. & Rissanen, A. J. 2015. Functional gene pyrosequencing reveals core proteobacterial denitrifiers in boreal lakes. *Frontiers in Microbiology,* 6:674.

Sanford, R. A., Wagner, D. D., Wu, Q. Z., Chee-Sanford, J. C., Thomas, S. H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K. K., Ritalahti, K. M., Nissen, S., Konstantinidis, K. T. & Löffler, F. E. 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proceedings of the National Academy of Sciences of the United States of America,* 109(48)**,** pp 19709-19714.

Schipper, L. A., Cameron, S. C. & Warneke, S. 2010a. Nitrate removal from three different effluents using large-scale denitrification beds. *Ecological Engineering,* 36(11)**,** pp 1552-1557.

Schipper, L. A., Robertson, W. D., Gold, A. J., Jaynes, D. B. & Cameron, S. C. 2010b. Denitrifying bioreactors-An approach for reducing nitrate loads to receiving waters. *Ecological Engineering,* 36(11)**,** pp 1532-1543.

Schipper, L. A. & Vojvodić-Vuković, M. 2000. Nitrate removal from groundwater and denitrification rates in a porous treatment wall amended with sawdust. *Ecological Engineering,* 14(3)**,** pp 269-278.

Schmid, M. C., Hooper, A. B., Klotz, M. G., Woebken, D., Lam, P., Kuypers, M. M. M., Pommerening-Roeser, A., op den Camp, H. J. M. & Jetten, M. S. M. 2008. Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. *Environmental Microbiology,* 10(11)**,** pp 3140-3149.

Schmidt, C. A. & Clark, M. W. 2013. Deciphering and modeling the physicochemical drivers of denitrification rates in bioreactors. *Ecological Engineering,* 60, pp 276-288.

SFS 2001:554. *Förordning om miljökvalitetsnormer för fisk- och musselvatten.* Stockholm: Miljö- och energidepartementet. (In Swedish).

Shapleigh, J. P. 2013. Denitrifying Prokaryotes. *In:* Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E. & Thompson, F. (eds.) *The Prokaryotes: Prokaryotic Physiology and Biochemistry.* Berlin, Heidelberg: Springer Berlin Heidelberg.

Shoun, H., Kim, D. H., Uchiyama, H. & Sugiyama, J. 1992. Denitrification by Fungi. *FEMS Microbiology Letters,* 94(3)**,** pp 277-281.

SMHI (2018). *Klimatdata*. Available at: http://www.smhi.se/klimatdata/meteorologi/temperatur/dataserier-med-normalvarden-1.7354 [2018-07-04]

SLU (2006). *Markinfo*. Available at: http://www-markinfo.slu.se/eng/climate/vegper.html [2018-07-04]

Song, B., Lisa, J. A. & Tobias, C. R. 2014. Linking DNRA community structure and activity in a shallow lagoonal estuarine system. *Frontiers in Microbiology,* 5:460.

Stein, L. Y. & Klotz, M. G. 2016. The nitrogen cycle. *Current Biology,* 26(3)**,** pp R94-R98.

Stoliker, D. L., Repert, D. A., Smith, R. L., Song, B., LeBlanc, D. R., McCobb, T. D., Conaway, C. H., Hyun, S. P., Koh, D.-C., Moon, H. S. & Kent, D. B. 2016. Hydrologic Controls on Nitrogen Cycling Processes and Functional Gene Abundance in Sediments of a Groundwater Flow-Through Lake. *Environmental Science & Technology,* 50(7)**,** pp 3649-3657.

Tiedje, J. M., Simkins, S. & Groffman, P. M. 1989. Perspectives on Measurement of Denitrification in the field Including Recommended Protocols for Acetylene Based Methods. *Plant and Soil,* 115(2)**,** pp 261-284.

Trauth, N., Musolff, A., Knoeller, K., Kaden, U. S., Keller, T., Werban, U. & Fleckenstein, J. H. 2018. River water infiltration enhances denitrification efficiency in riparian groundwater. *Water Research,* 130, pp 185-199.

van de Graaf, A. A., de Bruijn, P., Robertson, L. A., Jetten, M. S. M. & Kuenen, J. G. 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology,* 142(8)**,** pp 2187-2196.

Warneck, P. & Wurzinger, C. 1988. Product Quantum Yields for the 305 nm Photodecomposition of NO3 in Aqueous Solution. *Journal of Physical Chemistry,* 92(22)**,** pp 6278-6283.

Warneke, S., Schipper, L. A., Bruesewitz, D. A., McDonald, I. & Cameron, S. 2011. Rates, controls and potential adverse effects of nitrate removal in a denitrification bed. *Ecological Engineering,* 37(3)**,** pp 511-522.

Wessén, E., Söderstrom, M., Stenberg, M., Bru, D., Hellman, M., Welsh, A., Thomsen, F., Klemedtson, L., Philippot, L. & Hallin, S. 2011. Spatial distribution of ammonia-oxidizing bacteria and archaea across a 44-hectare farm related to ecosystem functioning. *ISME Journal,* 5(7)**,** pp 1213-1225.

Woehle, C., Roy, A.-S., Glock, N., Wein, T., Weissenbach, J., Rosenstiel, P., Hiebenthal, C., Michels, J., Schönfeld, J. & Dagan, T. 2018. A Novel Eukaryotic Denitrification Pathway in Foraminifera. *Current Biology,* 28(16)**,** pp 2536-2543.

Volokita, M., Belkin, S., Abeliovich, A. & Soares, M. I. M. 1996. Biological denitrification of drinking water using newspaper. *Water Research,* 30(4)**,** pp 965-971.

Xing, W., Li, J. L., Li, D. S., Hu, J. C., Deng, S. H., Cui, Y. W. & Yao, H. 2018. Stable-Isotope Probing Reveals the Activity and Function of Autotrophic and Heterotrophic Denitrifiers in Nitrate Removal from Organic-Limited Wastewater. *Environmental Science & Technology,* 52(14)**,** pp 7867-7875.

Yoshinari, T. & Knowles, R. 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. *Biochemical and Biophysical Research Communications,* 69(3)**,** pp 705-710.

Zhao, J. M., Feng, C. P., Tong, S., Chen, N., Dong, S. S., Peng, T. & Jin, S. L. 2018. Denitrification behavior and microbial community spatial distribution inside woodchip-based solid-phase denitrification (W-SPD) bioreactor for nitrate-contaminated water treatment. *Bioresource Technology,* 249, pp 869-879.

Zhu, I. & Getting, T. 2012. A review of nitrate reduction using inorganic materials. *Environmental Technology Reviews,* 1(1)**,** pp 46-58.

Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews,* 61(4)**,** pp 533-616.

Acknowledgements

I have been working at SLU since 2003 and have met many people that have meant a lot to me. I will try to mention only those who have been important for the outcome of this thesis:

Sara Hallin, when I wanted to be more involved in the research projects, learn more, take courses and write a thesis, you could have said no. Thank you for saying yes and believing in that I really could do it. I am proud of working in your group.

Jaanis Juhanson, thank you for saying “I guess yes” when you were asked to be my co-supervisor. It would have been much more difficult without your end-less patience in answering my questions.

Sari Peura, I am glad you wanted to join the supervisor group and support me. Your contribution during the last months was invaluable.

Robert Almstrand, thank you for all discussions and hands-on work while setting up the paper II experiment. I am sorry you had to go back to Gothenburg.

The MECO group, present and previous members of, my safe harbour on the journey. I do not write out all names, you know who you are .

The miNing group researchers, Frauke Ecke, Sara Hallin, Roger Herbert and Anders Widerlund. Thank you for generously sharing your knowledge, so many things were new and unknown to me when the project started.

The miNing group students, Maidul Choudhury, Lino Nilsson and Albin Nordström. It has been really fun working with you, discussing and learning new things. It was a privilege to directly take part in the other miNing sub-projects.

German Bonilla-Rosso and Valerie Hubalek, included but not explicitly mentioned above. I liked sharing practical work and writing with you.

Chris Jones, I am so glad we are both colleagues and friends.

For not negligible amounts of work with logistics, sampling, constructing and the handling of hundreds of litres of water, Robert Andersson, Johan Jonasson, Joel Segersten and Harry Winbjörk.

For creating a nice atmosphere to work in, all “Mykopat” colleagues and especially Rena Gadjieva, Katarina Ihrmark and Maria Jonsson. And at former “Mikro”, thanks especially to Elisabet Börjesson and Maria Erikson for friendship and GC support.

Sist men inte minst, Mats, Oskar och Anna. Tack för att ni står ut. Jag älskar er.