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# The effect of different carbon sources on reduction of nitrate in effluent from the mining industry

## Olika kolkällors inverkan på reduktion av nitrat i processvatten från gruvindustrin

Hanna Lindberg

## Abstract

**The effect of different carbon sources on reduction of nitrate in effluent from the mining industry**
*Hanna Lindberg*

Mine water effluent contains high levels of nitrogen due to residues from undetonated ammonium- nitrate based explosives. Excess nitrogen in aquatic ecosystems can cause eutrophication. Within a mining area, tailings and clarification ponds have the potential to reduce nitrogen levels by biological uptake of nitrogen into growing algae and denitrification in pond sediments. A previous study at the LKAB Kiruna mine investigated the potential nitrogen removal within the tailings and clarification ponds. The study showed that about 1-10 tonnes of nitrogen were removed each year, and that the removal by denitrification was limited by carbon.

The aim of this master thesis was to investigate if additions of different carbon compounds could improve the denitrification in sediment from the clarification pond at the LKAB Kiruna mine site. It was also of interest to see if the composition of the endogenous microbial community involved in nitrogen reduction changed after the treatments. Samples of sediment and pond water were collected in January 2014 and a laboratory experiment was set up where sediment and water was incubated with carbon additions under anoxic conditions. Three different carbon sources were tested: sodium acetate, hydroxyethyl cellulose and green algae. Pond water without additional carbon was used as a control. The sediment was incubated eight weeks at 20 °C with weekly water exchange and carbon addition. The removed water was analyzed to determine the amount of nitrogen removed. At start and after ending the incubation, potential denitrification in the sediment was determined with an enzymatic assay and the size of the genetic potential of nitrogen reduction was determined.

At start, the enzymatic assay showed that the potential denitrification rate in the sediment of the clarification pond at the LKAB Kiruna mine was not immediately enhanced by addition of carbon. However, during the incubation the removal of nitrate was enhanced by external carbon sources. Algae were a good carbon source, since the denitrifying community grew, the potential denitrification increased four times after incubation and the removal of nitrate was next to complete in the end of the incubation. The addition of cellulose also enhanced the denitrification activity to some extent and the abundance of genes coupled to denitrification increased. Further studies are needed to assess the practical use of external carbon sources like algae and plant material and how they would function in and potentially also affect a large, cold and complex system like the LKAB mining site.



Keywords: Denitrification, mine effluent, nitrogen, external carbon, sediment



*Department of Microbiology, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, ISSN 1401-5765*

# Referat

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*Hanna Lindberg*

Processvatten från gruvindustrin innehåller höga halter av kväve till följd av odetonerade ammonium- och nitratbaserade sprängämnen. Förhöjda halter kväve på grund av utsläpp av kväverikt vatten kan i akvatiska ekosystem leda till övergödning. Inom ett gruvområde har sedimentations- och klarningsdammar stor potential att reducera kvävemängder genom upptag av kväve i växande alger och genom denitrifikation i sedimenten. En studie gjord i LKABs gruva i Kiruna fann att 1-10 ton kväve årligen lämnar systemet via processer i sedimentations- och klarningsdammarna, samt att denitrifikationen var begränsad av kol.

Syftet med detta examensarbete var att undersöka möjligheten att öka denitrifikationen i sediment från klarningsdammen i Kiruna genom att tillsätta olika kolkällor. Även förändringar i mikroorganismsamhället i sedimenten på grund av koltillsatserna var intressanta att studera. Sediment och vatten provtogs i januari 2014 och ett experiment i laboratorieskala sattes upp. Vatten samt sediment inkuberades tillsammans med en kolkälla under anaeroba förhållanden. De koltillsatser som användes i försöket var natriumacetat, hydroxyetylcellulosa och grönalger. Vatten användes som kontroll. Inkubationen varade i åtta veckor i 20 °C, och varje vecka byttes vattnet ut och nytt kol tillsattes. Vattnet analyserades för att bestämma mängden nitrat som reducerats i och med de olika tillsatserna. För det ursprungliga sedimentet från klarningsdammen samt sediment efter inkubationen bestämdes potentiell denitrifikation och den genetiska potentialen för reducering av kväve analyserades.

Den potentiella denitrifikationen blev inte högre med tillsats av kol i det ursprungliga sedimentet från klarningsdammen vid LKABs gruva i Kiruna. Reduktionen av nitrat blev dock högre vid tillsats av kol under inkubationen. Tillsats av alger visade sig vara en god kolkälla, det denitrifierande mikroorganismsamhället växte, den potentiella denitrificationen ökade efter inkubationen och reduktionen av nitrat var näst intill fullständig i slutet av inkubationen. Tillsats av cellulosan ökade även det reduktionen av nitrat något och antalet av gener kopplade till denitrifikation ökade. Att tillsätta en kolkälla som alger eller växtmaterial till ett stort, kallt och komplex system som det vid LKAB:s gruvområde behöver dock utredas vidare för att kunna avgöra om tillskotten är praktiskt möjliga.

Nyckelord: Denitrifikation, gruvvatten utsläpp, kväve, externt kol, sediment

*Instutionen för Mikrobiologi, Sveriges Lantbruksuniversitet, SE-750 07 Uppsala,*
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## Preface

This master thesis of 30 credits has been written as a part of the Aquatic and Environmental Engineering Programme at Uppsala University and the Swedish University of Agricultural Sciences (SLU). This thesis was a part of a project called miNing, with the aim to remove nitrogen from mine effluent by passive and semi-passive solutions. The project is financed by Loussavaara Kiirunavaara AB (LKAB), Boliden Mineral AB and the Swedish Governmental Agency for Innovation Systems (VINNOVA). The thesis was conducted at SLU with Maria Hellman at the Department of microbiology as supervisor. Subject reviewer was Sara Hallin, also at the Department of microbiology. Examiner was Fritjof Fagerlund at the Department of Earth Sciences, Program for Air, Water and Landscape Sciences, Uppsala University.

A special thanks to Maria Hellman for the support and encouragement during this master thesis. I also want to thank Sara Hallin who has given valuable feedback and input during the project. I would also like to thank LKAB, for help and support with sampling. It would not have been possible to sample water and sediment a cold day in January without Ulf Nilsson, Rune Skarpsvärd and Malin Suup. Your encouraging attitudes and skills in repairing a malfunctioning Ekman grabber were invaluable.


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## Populärvetenskaplig sammanfattning

### Olika kolkällors inverkan på reduktion av nitrat i processvatten från gruvindustrin

*Hanna Lindberg*

Processvatten från gruvindustrin innehåller höga halter kväve som kommer från rester av odetonerade kvävebaserade spräng ämnen som används i gruvan. Grundvatten från gruvorna används inom gruvområdet och leds till sedimentations- och klarningsdammar innan överskottsvattnet leds vidare till närliggande vattendrag. Höga halter kväve i naturen kan leda till övergödning, algblomning och syrebrist. En studie visade att vattendrag som fått tillskott av kväverikt gruvvatten bland annat hade omkring 20 gånger mer alger i vattnet än närliggande vattendrag. Vissa kväveföreningar kan även vara giftiga för fisk och andra vattenlevande djur i höga halter.

Inom ett projekt kallat miNing undersöks olika passiva och semi-passiva metoder som kan reducera kvävemängderna till låga kostnader och lite underhåll. Inom gruvområdet har sedimentations- och klarningsdammarna potential att genom biologiska processer minska mängden kväve. En speciellt intressant process är denitrifikation som utförs av mikroorganismer i sedimenten under låga syreförhållanden. Denitrifikation är en process där nitrat, $NO_3^-$, i flera steg ombildas till kvävgas, $N_2$, som är den största beståndsdelen i luft. En tidigare studie gjord i LKABs gruva i Kiruna visade att dentrifikationen i klarningsdammen var begränsad av kol. De mikroorganismer som utför denitrifikationen är nämligen beroende av en organisk kolkälla till skillnad från växter och alger som använder luftens koldioxid som kolkälla. En annan process som minskar mängden kväve under låga syreförhållanden kallas anammox. De mikroorganismer som utför anammox behöver dock inte någon organisk kolkälla utan kan använda koldioxid.

Detta examensarbete hade som syfte att undersöka hur olika typer av kol påverkade reduktionen av nitrat i kväverikt gruvvatten. Det mikrobiella samhället i sedimentet från klarningsdammen skulle även det undersökas, samt hur de olika koltillskotten skulle påverka det. Provtagning av sediment samt vatten i LKABs klarningsdamm vid gruvan i Kiruna gjordes i januari 2014. Sedimentet placerades sedan, tillsammans med vatten och en kolkälla, i 500 mL glasflaskor. De kolkällor som användes i försöket var natriumacetat (NaAc), hydroxyetylcelullosa (HEC), samt grönalger. NaAc är en lättillgänglig kolkälla som mikroorganismerna kan ta upp direkt. HEC, som är en cellulosa, skulle representera ett nedbrutet växtmaterial och alger är en kolkälla som eventuellt skulle kunna odlas i klarningsdammen. Vatten användes som kontroll. Glasflaskorna fick stå i 20 °C under åtta veckor och varje vecka byttes vattnet ut och nytt kol tillsattes. Vattnet analyserades för att kunna bestämma mängden nitrat som försvunnit varje vecka. För sediment från klarningsdammen samt efter inkubationen bestämdes potentiell denitrifikation dessutom gjordes mikrobiella analyser. Potentiell denitrifikation ger en hastighet för ombildningen av nitrat till lustgas (som är steget innan kvävgas bildas) som beror av antalet aktiva mikroorganismer och typen av kol

som tillsätts. Analysen av det mikrobiella samhället gjordes med qPCR vilket är en metod där antalet av en viss gen i en bestämd mängd sediment beräknas. För det mikrobiella samhället undersöktes dels det totala antalet bakterier och för det denitrifierande samhället användes gener som kodar för specifika enzym vid de olika stegen för denitrifikationen. Den genetiska potentialen för anammox undersöktes också.

Den potentiella denitrifikationen i det ursprungliga sedimentet var densamma oberoende av om kol tillsattes eller inte, vilket visar att mängden kol inte begränsade organismerna i sedimentet vid provtagningstillfället. Vattenanalyserna gjorda under inkubationen visade dock att tillsatt kol ökade reduktionen av nitrat. NaAc hade nästan fullständig reduktion av nitrat varje vecka, och tillsatsen av alger hade det de två sista veckorna. Tillsatsen av cellulosan, HEC, gav endast något bättre rening än tillsatsen av vatten. Bestämningen av den potentiella denitrifikationen i sedimenten efter inkubationen visade att alger var den enda koltillsatsen som gav en ökning jämfört med den potentiella denitrifikationen i det ursprungliga sedimentet. Ökningen var omkring fyra gånger större. Även den totala mängden bakterier och det denitrifierande samhället var större i sediment som fått alger jämfört med sediment innan inkubationen. Tillsatsen av cellulosan gav även det en ökning av vissa av gener kopplade till denitrifikationen. Det fanns en genetisk potential för anammox i sedimenten från klarningsdammen och denna fanns även kvar i alla sediment efter inkubationen.

Slutsatsen av detta försök var att denitrifikationen, och reduktionen av nitrat kan förbättras med tillsats av kol. Tillsats av alger har en mycket hög potential att öka reduktionen av kväve, men faktorer som begränsar algtillväxten i klarningsdammen måste utredas. Kanske är det inte möjligt att öka mängden alger utan att även påverka de vattendrag som är nedströms dammen. Under den begränsade tid som försöket pågick visade tillsats av HEC att växtmaterial skulle kunna förbättra denitrifikationen, men även här behövs ytterligare studier för att avgöra om reduktionen av kväve även skulle vara möjlig i en större skala.

## Abbreviations

C - Carbon

DNRA- Dissimilatory nitrate reduction to ammonium

DOC- Dissolved organic carbon

N - Nitrogen

$NH_3$ - Ammonia

$NH_4^+$ - Ammonium

$N_2$ - Dinitrogen gas

$N_2O$ - Nitrous oxide

NO - Nitric oxide

$NO_2^-$ - Nitrite

$NO_3^-$- Nitrate

P - Phosphorous

PDA - Potential denitrification activity

$PO_4$-P - Phosphate phosphorous

qPCR - Real-time quantitative polymerase chain reaction

TC - Total carbon

TN - Total nitrogen

TOC - Total organic carbon

## CONTENTS

# 1. INTRODUCTION

Mine water effluent contains high levels of inorganic nitrogen (N) compounds at concentrations comparable with those found in municipal sewage water. Considering that lakes and streams in northern Sweden have the lowest N levels in Sweden, less than 0.3 mg N $L^{-1}$, additional N can have a considerable impact on these ecosystems (Havs- och vattenmyndigheten, 2014). High levels of N in effluents can lead to eutrophication, which in return could lead to algae blooms and hypoxia in receiving waters (Naturvårdsverket, 2012). Certain N species are also toxic to aquatic life, and high levels of ammonia ($NH_3$) or nitrite ($NO_2^-$) could even be lethal (Huey and Beitinger, 1980; Alonso and Camargo, 2003).

One of the Swedish environmental quality objectives adopted by the Swedish Parliament is zero eutrophication and in the European Union the water quality of natural water bodies are regulated by the Water Framework Directive (WFD) (Naturvårdsverket, 2012; European Union, 2000). The WFD states that by 2015 the status of all surface waters greater than 0.5 $km^2$ must at least be of good status. If a water body does not have a good status the concerned country need to restore the water body or ask for an extension (European Union, 2000). Restoration might be necessary when N rich mine effluent alter the balance in recipient water bodies.

Possibilities to reduce N in mine effluent by passive and semi- passive methods, e.g wetlands and barrier systems, are investigated within a project called miNing conducted in collaboration between Uppsala University, Swedish University of Agricultural Sciences, Luleå University of Technology and Rock Tech Centre and financed by Loussavaara Kiirunavaara AB (LKAB), Boliden Mineral AB and the Swedish Governmental Agency for Innovation Systems (VINNOVA). The methods investigated for N removal rely on enhancing microbial activities such as nitrification, denitrification and anammox. Within a mine area the tailings and clarification ponds have potential conditions for naturally reducing N before the water is discharged. The water bodies have large surface areas and long water resident times which could offer potential for biological uptake of N into growing algae and denitrification in pond sediments. A limiting factor and a challenge for biological processes in northern Sweden is the cold climate (the water temperature is only about 0.2-15 °C in the ponds at the mine site) and nutrient limitations (Ecke et al., unpublished).

As a part of miNing, the tailings and clarification ponds at the LKAB Kiruna mine were previously shown to have the capacity to remove 1-10 tonnes N each per year in the tailings and clarification ponds by phytoplankton uptake and denitrification (Ecke et al., unpublished). The study also showed that the potential denitrification was 10-40 times higher in the clarification pond than in the tailings pond, which corresponded with the overall bacterial community being about 10 times more abundant. The clarification pond also had more algae growth and more organic carbon (C) content than the tailings pond. Nevertheless, the organic carbon content in the clarification pond was not high enough to support sufficient N-removal by denitrification and it was concluded that the process was limited by C (Ecke et al., unpublished).

### 1.1 OBJECTIVE

The objective was to investigate if the denitrification in the sediment of the clarification pond at LKAB Kiruna mining site can be enhanced by different carbon additions. To study the potential of carbon addition to the pond sediment three different C sources were examined. Sodium acetate (NaAc) was chosen as a C source which can be utilized by the organisms directly, whereas hydroxyl ethyl cellulose (HEC), a cellulose derivative, represented decaying plant material and algae is a potential C source that could be cultivated in a clarification pond. It was also investigated if the size of the microbial communities involved in different N-removal processes change with additions of different C types.

The experimental parameters such as temperature, volumes and time chosen, were not supposed to exactly mimic the local conditions or the whole natural system. The objective was only to examine the potential of the different C additions.

## 2. BACKGROUND

### 2.1 NITROGEN SOURCES IN MINING

The major source of N in the mine water is explosives like Ammonium Nitrate Fuel Oil (ANFO), emulsion explosives and watergel-based explosives, which all contain large amounts of ammonium nitrate ($NH_4NO_3$ ) (Revey, 1996). ANFO can be described as 94% $NH_4NO_3$ and 6 % fuel oil (here $CH_2$) with the following reaction (reaction 1) when detonating (Morin and Hutt 2009):

$$3NH_4NO_3 + CH_2 \rightarrow 7H_2O + CO_2 + 3N_2 + heat \qquad (1)$$

Reaction (1) shows that the complete detonation would result in all N species being converted to dinitrogen ($N_2$) which is an inert gas and the major component of air. Some of the explosives do not fully detonate or in some cases even fail to do so (Morin and Hutt, 2009). Undetonated explosives containing soluble N species like ammonium ($NH_4^+$) and nitrate ($NO_3^-$) are therefore left on the particle surfaces or dissolve in the surrounding ground water (Revey, 1996). Two examples of why detonations fail are cut offs and dead pressing (Revey, 1996). Cut offs are caused by neighbouring detonations which damage the detonating cord or cause rock movements that separate the explosives. Dead pressing occurs when the density of the explosives exceed a critical limit due to too great of a shock from neighbouring detonations. Spillage during charging is another source of N contamination (Revey, 1996). Other sources of N contaminants in mining are cyanide used in gold extraction, transformations of amines in flotation circuits and pH regulation agents (Mattila, 2007).

In most mines the N-contaminated groundwater is pumped from the mine to prevent flooding and is used in the iron process plant where N is also washed out from the rock surface (Mattila, 2007). The water is lead to tailings and clarification ponds from which water is partially recirculated to the process plants. The excess water is discharged from the clarification pond to local surface water recipients (Chlot et al., 2013). Effluent rich

in N also leak from barren rock piles, especially during spring flood and heavy rainfall events (Mattila, 2007).

Mass flow calculations showed that 15- 19% of the used explosives in LKAB Kiruna mine was undetonated and brought to the surface with the ore or dissolved in the mine water (Forsberg and Åkerlund, 1999). As an example, in the Kiruna mine about 20-30 tons of explosives detonate each day (LKAB, 2014). Work has been carried out to minimize the amount of undetonated explosives by educating the charging personnel and by making more efficient explosives. With these improvements there has been a reduction of undetonated explosives per ton ore, but due to production increase the amount N released to surrounding water is still about the same (Ecke et al., 2011).

### 2.2 ENVIRONMENTAL EFFECTS OF NITROGEN POLLUTION

Nitrogen is an essential compound for all life and required in large amounts. Most living organisms cannot utilize the stable $N_2$ in the atmosphere, instead reactive N e.g. $NH_4^+$, $NO_3^-$ or organic nitrogen is required (Thamdrup, 2012). According to Thamdrup (2012), the fixed N in the biosphere corresponds to 0.1 % of the $N_2$ pool, and N is therefore often a limiting nutrient in nature.

In aquatic ecosystems not only N can be limiting for growth but also phosphorous (P). Freshwater systems are generally considered to be limited by P (Schindler, 1977), but a review paper by Sterner (2008) propose that most lakes are co-limited by N and P and also in some cases iron (Fe) or other nutrients. Freshwater systems have also been shown to be limited by N (Jansson et al., 2001). By increasing the amount of N released to freshwater systems by mine effluent, alterations of the aquatic ecosystem can occur as well as eutrophication which in return can lead to hypoxia (Havs- och vattenmyndigheten, 2014). A study done by Chlot et al. (2013) showed that two mine site recipients had affected nutrient regimes with elevated N and P concentrations compared to nearby control lakes. The same study also showed that phytoplankton biomass was ~20-25 times higher in the recipient lakes than in the reference lakes, and the diversity of phytoplankton species were lower (Chlot et al., 2013).

Excess N does not only cause changes in water quality and cause eutrophication, but some N species are toxic to aquatic life. Nitrite ($NO_2^-$) can turn hemoglobin to methemoglobin, which is unable carry oxygen to cells and can therefore lead to tissue anoxia and death to fish and other aquatic organisms (Huey and Beitinger, 1980). Lethal levels of $NO_2^-$ range from 0.7 mg $L^{-1}$ to 27 mg $L^{-1}$ depending on fish species and water chemistry, with rainbow trout as the most sensitive fish studied (Huey and Beitinger, 1980). Ammonia ($NH_3$) which is in equilibrium with $NH_4^+$ is also toxic to fish and cause gill ventilation, hyperexcitability, convulsion and death (Alonso and Camargo, 2003). Ammonia is also toxic to other aquatic organisms like freshwater mussels and other invertebrates (EPA, 2009). The limit for N species in fish and mussel waters in Sweden is regulated by Act (2001:554) (Table 1). The same regulation also states guideline values which are the preferred values.

***Table 1.*** *Limit values and guideline values for $NO_2^-$, $NH_3$ and $NH_4^+$ in fish and mussel waters (Act 2001:554)*

| N species | Limit value (mg N $L^{-1}$) | Guideline value (mg N $L^{-1}$) |
|---|---|---|
| $NO_2^-$ | - | 0.03 |
| $NH_3$ | 0.025 | 0.005 |
| $NH_4^+$ | 1 | 0.2 |

### 2.3 REDUCTION OF NITROGEN SPECIES

The explosives used in the mine have a $NH_4^+$:$NO_3^-$-ratio of 1:1, and the ratio in the water effluent would therefore be considered to be the same (Chlot et al., 2013). However, biotic and abiotic transformation of N occurring at the mine site reduce the $NH_4^+$ level to just a few percent of total nitrogen (TN), whilst $NO_3^-$ increases to about 90 % of TN in the effluent (Chlot et al., 2013). There are several natural ways that N species in the mine effluent can be transformed and also reduced (Ecke et al., 2011; Figure 1). The most important processes will be presented below and these are physical, chemical and biological such as ammonia volatilization, uptake by macrophytes (aquatic plants), sedimentation processes and microbial nitrogen transformations. The N reducing processes can occur both in the mine, in the process plant, in the waste rock deposits, tailing impoundments and clarification ponds.

Nitrogen reducing processes also occur further downstream, in the recipients. Saunders and Kalff (2001) compared the different mechanisms retaining and removing N in streams, lakes and wetlands and most efficient was denitrification followed by sedimentation and uptake of macrophytes. The authors state that although sedimentation and uptake of macrophytes only play a small role in reducing N, the processes significantly contribute both directly and indirectly to the N cycling in freshwaters.

#### *2.3.1 AMMONIA VOLATILIZATION*

Ammonia volatilization is a physical process where $NH_3$ is lost to the atmosphere. $NH_3$ is relatively volatile and is in equilibrium with $NH_4^+$ where the pH and temperature determines the fraction of free $NH_3$. At a mine site, volatilization could at a mine site occur in tailings and clarification ponds and also in the process plant and at surfaces of ore and waste rock (Ecke et al., 2011). Ecke et al. (2011) propose that ammonia volatilization could be a significant N-sink in mine process water considering the process water has a temperature of ~20°C and slightly alkaline pH.

#### *2.3.2 UPTAKE BY MACROPHYTES AND SEDIMENTATION*

Macrophyte and microbial uptake of N is referred to as assimilation, which are biological processes that convert inorganic N, e.g. $NH_4^+$ and $NO_3^-$, to organic compounds that serve as building blocks for cells and tissues (Vymazal, 2007). Macrophyte potential uptake of N and also storage is limited by growth rate and concentration of nutrients in the plant tissue (Vymazal, 2007). In temperate climates the

N uptake is highest in the spring and summer, and during fall the shoots die and most of the biomass decompose (Vymazal, 2007).

One way to permanently remove N with the macrophytes is to harvest the plants (Fox et al., 2008). Even if the macrophytes are not harvested, they play an important role in reducing N (Hill, 1979). When the biomass decomposes, the N and C released are in organic form and are readily utilized by microorganisms in contrast to inorganic forms of C and N (Hill, 1979). The simultaneous release of N and C enhances denitrification that otherwise often is carbon limited (Sirivedhin and Gray, 2006). According to Reddy et al. (1989), macrophytes also offer an ideal environment for microorganisms that contribute to N removal by nitrification and denitrification, and the microorganisms are found in high numbers on roots and in the rhizosphere compared to surrounding sediments and water. The microbial activity is also higher in then root zone of macrophytes (Ruiz-Rueda et al., 2009).

In the deeper sediments, where the decomposition rate is negligible due to low oxygen levels, a permanent removal of N occurs. Westrich and Berner (1984) showed that organic matter originating from algae is more easily degradable than organic matter of terrestrial origin derived from vascular plants. The plants contain high amounts of cellulose, lignin and waxes which are biologically resistant and hence harder to degrade and the rate of remineralisation is lower (Westrich and Berner, 1984).

### *2.3.3 MICROBIAL TRANSFORMATIONS*

Microbial N transformations play an important role in the reduction of N, with denitrification and anaerobic ammonium oxidation (anammox) as the most important processes as they permanently remove N as $N_2$ emitted to the atmosphere (Saunders and Kalff, 2001; Vymazal, 2007). The N cycle is complex, particularly the biochemical N cycle where the transformations are carried out by distinct groups of microorganisms (Thamdrup, 2012; Figure 1). The processes that will be discussed in this report and that are the most important in the reduction of N in the mine water are nitrification (the combined process of ammonia oxidation and nitrite oxidation in Figure 1), denitrification and anammox. Dissimilatory reduction of nitrite to ammonia (DNRA) will also be discussed as this is an alternative route for nitrite reduction, but not resulting in N removal.

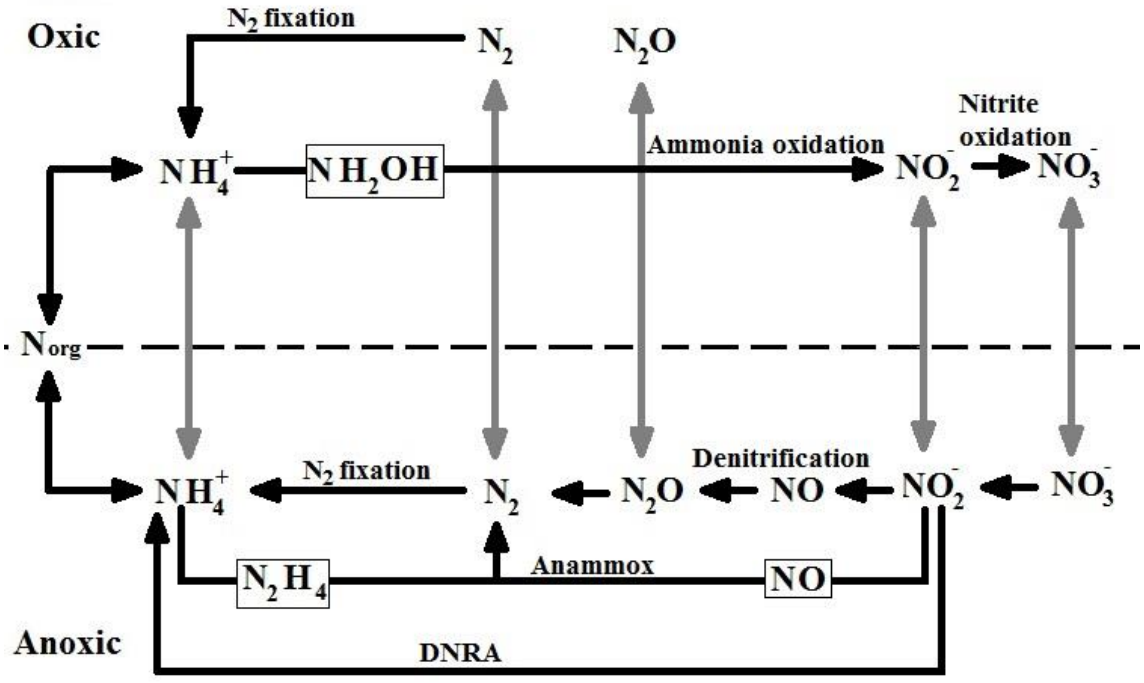


***Figure 1.*** *The biochemical N cycle (redrawn with modifications from Thamdrup, 2012).*

Nitrification by bacteria and archaea plays a central role in the biological N cycle since it converts $NH_3$ (which is in equilibrium with $NH_4^+$) to $NO_3^-$ which is the substrate for denitrification (Prosser, 1989). It is an aerobic process with optimum at mesophilic temperatures (25-35 °C) and neutral to alkaline pH, and the process can be summarized as (reaction 2) (Prosser, 1989; Vymazal, 2007):

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

The first step of nitrification is the oxidation of $NH_3$ to $NO_2^-$ and it is carried out in two steps with the intermediate hydroxylamine ($NH_2OH$) (Prosser, 1989). Oxidation of $NH_3$ to $NH_2OH$ is catalyzed by the enzyme ammonia monooxygenase (Hollocher et al., 1981; Rotthauwe et al., 1997). Hydroxylamine is further oxidized to $NO_2^-$ with the enzyme hydroxylamine oxidoreductase (Prosser, 1989). The organisms involved in oxidizing $NH_3$ to $NO_2^-$ are generally considered chemolithotrophic, meaning that they gain their energy from oxidizing inorganic compounds, however some gain energy from organic compounds (Prosser, 1989; Vymazal, 2007; Schmidt et al., 2003; Tourna et al., 2011). The last step, nitrite oxidation, is done by bacteria carrying the enzyme nitrite oxidoreductase that catalyze the conversion of $NO_2^-$ to $NO_3^-$ (Prosser, 1989). The nitrite oxidizers are facultative chemolithotrophs and they can gain energy from both organic compounds and $NO_2^-$ (Vymazal, 2007).

Denitrification refers to the process where $NO_3^-$ is reduced via numerous steps to $N_2$ (Knowles, 1982). The first step is the reduction of $NO_3^-$ to $NO_2^-$, which is further reduced to nitric oxide (NO) and nitrous oxide ($N_2O$) and the final product will be $N_2$ if the process is complete (Knowles, 1982). Bacteria and archaea involved in denitrification use the nitrogen oxides as electron acceptors when oxygen is not present (Zumft, 1997).

The organisms carrying out the denitrifying process are very diverse both taxonomically and biochemically. Most have the enzymes, reductases, to reduce $NO_3^-$ completely to $N_2$ (Knowles, 1982). However, there are also organisms that only carry out a few or just one step in the pathway and some organisms therefore produce NO or $N_2O$ as the final product (Knowles, 1982). There are also denitrifiers that do not have the $NO_3^-$ reductase and they use $NO_2^-$ as an external electron acceptor (Knowles, 1982). Other bacteria only have $N_2O$ reductase and act as $N_2O$ sinks (Zumft, 1997).

Each of the enzymes catalyzing the steps in the pathway is encoded by specific genes (Figure 2). The first step, $NO_3^-$ to $NO_2^-$, is carried out by two types of reductases encoded by the genes *narG* and *napA*. These genes are found not only in denitrifying organisms, but in nitrate reducing bacteria as well (Zumft, 1997). The genes *nirS* and *nirK* encode for the enzymes catalyzing the step from $NO_2^-$ to NO, and the vast majority of microorganisms capable of this reduction have one or the other (Zumft, 1997). The step between NO and $N_2O$ is associated with the Nor enzymes, which are encoded by the two variants of *norB* genes (Zumft, 1997). As with *narG* and *napA*, these enzymes can be found also in other organisms than denitrifiers (Zumft, 1997). The last step of the denitrification is the reduction of $N_2O$ to $N_2$ and this is performed by the enzyme nitrous oxide reductase, encoded by *nosZ*. The nitrous oxide reductase can be divided into two clades and the corresponding genes are denoted *nosZ*I and *nosZ*II (Jones et al., 2013).

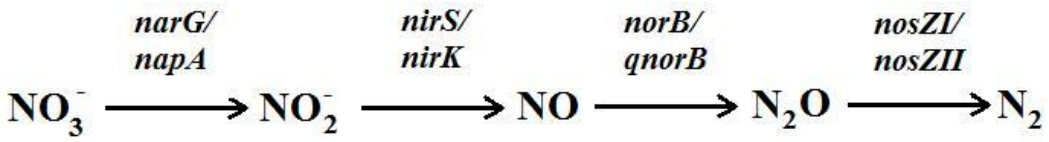


***Figure 2****.The steps of denitrification and the genes encoding for the enzymes catalyzing each step.*

The complete denitrification reaction can be summarized as (reaction 3) (Hiscock et al., 1991):

$$5(CH_2O) + 4\,NO_3^- + 4H^+ \rightarrow 5\,CO_2 + 2\,N_2 + 7\,H_2O \qquad (3)$$

Reaction (3) shows that denitrification is dependent on C compounds, and the denitrifying organisms are mostly heterotrophs meaning that they need organic C

(Knowles, 1982). The amount of available organic C is one of the most important factors controlling the activity of the heterotrophic denitrifying bacteria and archaea. Other factors influencing the denitrification are e.g. dioxygen ($O_2$) levels, pH and temperature (Knowles, 1982). The presence of $O_2$ represses denitrification, since the organisms prefer to respire with oxygen, but different organisms and also the different reductase show varying sensitivity to $O_2$. This sometimes leads to different end products (e.g. $N_2O$ or $N_2$) depending on the $O_2$ content (Knowles, 1982). pH also represses different reductases at different levels, the optimum pH for complete denitrification is 7-8, and at low pH more $N_2O$ tend to be produced (Knowles, 1982). The temperature also affects the denitrification rate, decreasing the temperature decreases the denitrification rate, but even at 0-5 °C denitrification is measurable (Knowles, 1982).

Another process which reduces $NO_2^-$ is dissimilatory nitrite reduction to ammonia (DNRA), in which $NO_2^-$ is reduced to $NH_4^+$ (Sgouridis et al., 2011). The DNRA process is strictly anaerobic and occurs during the same conditions as denitrification, low oxygen levels as well as available $NO_2^-$ and C (Sgouridis et al., 2011). However an important difference is that denitrification removes N from the system whereas DNRA only transforms N to a more easily biological available N specie (Sgouridis et al., 2011).

In addition to denitrification, N can be permanently removed by the process anammox which combines $NH_4^+$ and $NO_2^-$ to $N_2$ by the following reaction (reaction 4) (Vymazal, 2007):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O \qquad (4)$$

Anammox is performed by a group of slow growing bacteria and is a quite new discovery in the different microbial pathways of the N cycle (Straus et al., 1999). These bacteria are autotrophs, i.e. fix C from carbon dioxide, meaning that they are not dependent on organic C compounds like the denitrifiers. Anammox has its optimum at neutral pH and temperatures at 20-43°C and is inhibited by oxygen and high $NO_2^-$ concentrations (Strous et al., 1999). Although anammox bacteria have been found in many different environments it is still not established how much this process contribute to $N_2$ formation in e.g. wetlands (Vymazal, 2007; Zhu et al., 2010). The process however contributes substantially to N removal in marine sediments (Dalsgaard et al., 2005).

# 3. METHODS

## 3.1 FIELD SITE AND SAMPLING

Samples of sediment and water were taken from three locations in the clarification pond at the LKAB Kiruna mine site (Figure 3).


***Figure 3****. Aerial photograph of the LKAB Kiruna mine site. a) The mine site and the tailings and clarification ponds. b) The three sampling stations for water and sediment samples, indicated as white dots, in the clarification pond (hitta.se, 2014).*

The samples were collected in January 2014. The air temperature was -24 °C and the samples were taken via auger holes through the ice cover. Surface water, in total 40 L, was collected with a plastic beaker from all three locations, mixed together and distributed in two 25 L water containers. Samples of the uppermost 1-3 cm of the sediment were collected from all three locations with an Ekman grabber and put in plastic bags. The sediment samples were let to settle at 4 °C overnight so that excess water could be removed, and were transported to the laboratory in a cooler bag. Water was transported frozen except for 2 L water which was analyzed within 24 h. At arrival to the laboratory, the water was thawed and aliquoted into 4 L portions and re-frozen.

### 3.2 Analyzes of water and sediment

The water was analyzed at the SWEDAC accredited Geochemical laboratory at SLU. pH, $NH_4^+$-N, $NO_2^-$+$NO_3^-$ -N, total nitrogen (TN), total organic carbon (TOC), dissolved organic carbon (DOC) and phosphate- P ($PO_4$-P) were determined in the water, for methods and instruments see Table 2.

***Table 2.*** *Methods and instruments used in analyzing the water chemistry parameters*

| Parameter | Method | Instrument |
|---|---|---|
| pH | SS- EN ISO 10523:2012, Unaerated 25 °C, range 4-7 | Sampler changer Methrom 855 |
| $NH_4^+$-N | Bran & Luebbe method No G-171-96 for AAIII | Bran & Luebbe Autoanalyzer 3 |
| $NO_2^-$+$NO_3^-$ -N | SS- EN ISO 13395:1996 modified for Bran & Luebbe Method No. G- 287-02 for AAIII | Bran & Luebbe Autoanalyzer 3 |
| TN | SS-EN 12260:2004 | Shimadzu TOC-VCPH with TNM-1 module and automatic sample changer ASI-V |
| $PO_4$-P | Bran & Luebbe Method No. G-175-96 for AAIII | Bran & Luebbe Autoanalyzer 3 |
| TOC and DOC | SS-EN 1484 vers. 1<br><br>(DOC samples filtered through 0.45 µm filter) | Shimadzu TOC-VCPH with TNM-1 module and automatic sample changer ASI-V |

The water content in the sediment was determined by drying in a furnace at 105 °C over-night and measuring the difference in weight before and after drying. The total amount of carbon (TC), TN and TOC was determined in the sediment by the Soil laboratory at SLU with the LECO® CNS-2000 Carbon, Nitrogen and Sulphur Analyzer according to manufacturer's instructions.

### 3.3 MICROCOSMS

#### *3.3.1 SET UP*

In the laboratory the sediment was once more left over-night to settle at 2 °C and excess water was removed. The sediment was examined visually and two types of sediment could be distinguished. From one sampling station the sediment was black and had more roots, whilst the other two locations had more brown and claylike sediment. Bigger sticks and stones were removed and the sediment from all sampling stations were combined and homogenized by mixing twice in a kitchen blender.

The experiment was set up in microcosms (Hallin et al., 1996; Ragab et al., 1992; Ingersoll and Baker, 1998; Robins et al., 2000), 500 mL glass bottles with gas-tight screw-caps with butyl rubber septa. In each bottle, 100 g of sediment and 300 g of water

from the clarification pond were combined. Via a needle trough the septa connected to tubing, the gas produced in the microcosms was lead to a measuring cylinder with water lock (Figure 4). The gas volume was collected and measured as a proxy for microbial activity in the microcosms during the experiment.

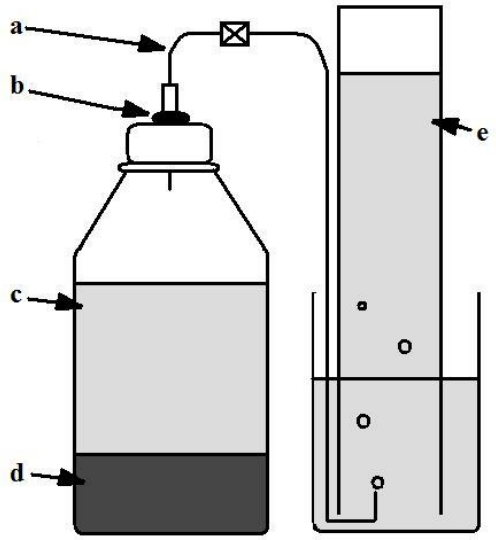


***Figure 4.*** *Schematic picture of the microcosm setup. (a) Needle connected to tubing and valve. (b) Butyl rubber septum. (c) Water from clarification pond. (d) Sediment. (e) Graduated glass cylinder to measure gas production.*

### *3.3.2 CARBON SOURCES*

Three different carbon sources with increasing complexity were used to add carbon to the systems. The simplest C source was sodium acetate (NaAc) directly available for the microorganisms. NaAc is the sodium (Na) salt of acetic acid ($CH_3COOH$) with the total formula $C_2H_3NaO_2$. The more complex C source was hydroxyethyl cellulose (HEC), a polymer with a molecular weight of about 90 000 g $mol^{-1}$ and a C fraction of about 0.53 (Emmerich, Pers. Comm.). As the most complex carbon source, the fresh water green algae *Pseudokirchneriella subcapitata* (previously named *Selensastrum carpricornutum*) was chosen. The microcosms are hereafter referred to as the substrate they had been amended with, e.g. microcosms having received water during the experiment will be referred to as Water. The sediment before the incubation will be referred to as Start.

The same amount of C was added to each microcosm. The addition was in excess so neither the denitrification or growth of microorganisms would be limited by carbon. For assumptions and calculations of the amount of C needed in each microcosm and preparation of NaAc and HEC solutions, see Appendix I.

Carbon content in the algae was estimated by drying the algae at 105 °C over- night, and then burning at 550 °C over- night and by calculating the loss of weight organic matter content could be determined. For simplicity the organic matter content was assumed to be C content. To avoid the need to determine carbon content by burning for every new batch of algae used, optical density (OD) in the algae solution was determined as a function of carbon content in algae, see Appendix I.

The green algae were grown in medium as described in EPSAG (2013) with stirring and aeration. A carbon source for the algae was added, 25 mg/mL sodium bicarbonate ($NaHCO_3$) to enhance growth. An antibiotic, 100 µg/ mL Kanamycin, was also added so not to add bacteria to the microcosms that would not be naturally present. The pH in the algae solution was adjusted to 7.2- 7.6 by addition of potassium hydroxide (KOH).

Preparation of the algae before addition to the microcosms included washing with water to remove the antibiotics and the growth medium, concentrating the algae to prevent dilution of the added pond water, determining the carbon content and lysing (meaning breaking the cell membrane) the algae. Washing and concentrating the algae was done by centrifugation; the algae formed a pellet at the bottom of the flask, the supernatant was removed with vacuum suction and the remaining algae were resuspended in autoclaved deionized water. The procedure was repeated four times and by reducing the amount of water used in each step the algae were concentrated. Centrifugation was done at maximum speed using the Beckman JC-H6 with a swing- out rotor during 10 minutes and relative centrifugal force (rcf) of 4 000 xg for water volumes of about 400 mL, Beckman J2-HS with rotor JA-14 during 15 minutes at rcf of 14 000 xg when water volume was less than 200 mL and Jouan CR322 centrifuge with a swing-out rotor and rcf 3 000 xg for 10 minutes when using 50 mL tubes.

Since the experiment was performed during a limited period of time, the algae were lysed so that the organic matter in the algae would be more easily available to the microorganisms. The algae solution was lysed after washing and concentrating by beating portions of 1 mL with 0.15 g glass beads (2 mm in diameter) in 2 mL tubes using FastPrep® instrument (MP Biomedicals) for 40 seconds at speed 6.0 m $s^{-1}$. After bead beating, light microscopy (400x magnification) was used to verify lysing of the cells.

### *3.3.3 OPERATION OF THE MICROCOSMS*

At the start-up of the experiment, the microcosms were prepared as described in section 2.2.1, with three replicates of each of the different carbon treatments and with deionized water as a control. Each microcosm had additions of $K_2HPO_4$ to a final concentration of 20 mM as a buffer to stabilize pH at 7.4. Water, NaAc and HEC were added in the same volume of solution and the carbon addition with NaAc and HEC were 49 mg C. Algae was added as well, but due to shortage of material only 45 mg C was added. Finally the atmosphere in the bottles was changed to $N_2$ by alternating between vacuum and $N_2$-gas three times. The microcosms were kept at 20 °C except for when water was exchanged which was done at room temperature (about 22 °C).

The water in the bottles was exchanged weekly. The valve in the tubing was closed to prevent disturbance of the gas volume in the measuring cylinders and the needle was withdrawn from the septa in the lid. Water withdrawal was done by vacuum suction and the water was sent for analysis where the concentrations of TOC, pH, $NH_4$-N and $NO_2^-$ +$NO_3^-$ -N were determined. Analyzes were done as described in section 3.2. The same amount of water withdrawn from the microcosms was added to each bottle. The added water was water sampled from the clarification pond and had been stored frozen. The water exchange was done the first three weeks in a fume cupboard but moved to a laminar flow cabinet to reduce risk of contaminating the microcosms.

As in the start, 49 mg C was added weekly to the NaAc and HEC microcosms, with a corresponding addition of water to the control bottles and algae was added according to Table 3. Adding the algae solution by calculating C content by OD was done at the beginning of the experiment. When the algae solution used during week two and three was burned to determine the exact carbon amount the OD and C content did not agree with the calculations done. The algae solutions added during week four to seven was calculated after determining C content by burning.

***Table 3.*** *Weekly calculated and determined carbon content for addition of algae*

| **Week** | **Algae (Calculated mg C)** | **Algae (Determined mg C)** |
|---|---|---|
| Start up | 45 | - |
| 1 | 49 | - |
| 2 | 49 | 12 |
| 3 | 49 | 12 |
| 4 | - | 49 |
| 5 | - | 49 |
| 6 | - | 49 |
| 7 | - | 49 |

Buffer was added as well the second week but not thereafter, due to unwanted precipitation that formed in the microcosms when the buffer was added. After change of water and C additions, atmosphere was exchanged to $N_2$ as described above and the tubing connected to the measuring cylinder was attached again. When necessary the water locks were refilled due to evaporation or redone due to a large amount of gas produced.

After nearly eight weeks, the sediments in the different microcosms were sampled and analyzed to determine water content and TOC. In addition, potential denitrification activity and abundance of different groups involved in inorganic N-cycling was determined.

### 3.4 POTENTIAL DENITRIFICATION ACTIVITY

Potential denitrification activity (PDA) determines the rate of which the microorganisms turn $NO_3^-$ to $N_2O$ by denitrification under optimal conditions. During the analysis the conditions for denitrification are optimized so that the amount of enzymes active in the denitrification and the environmental conditions investigated are the rate-limiting parameters (Pell et al., 1996). PDA was done using the acetylene inhibition technique without chloramphenicol as described by Pell et al. (1996) with some modifications. In short, sediment slurry was incubated with nitrate and carbon under anoxic conditions with added acetylene gas ($C_2H_2$). The $C_2H_2$ inhibits the formation of $N_2$ by blocking the nitrous oxide reductase performing the last step of denitrification, and only $N_2O$ is formed and accumulated. The concentration of the $N_2O$ can then be determined by gas chromatography.

The denitrification rate was determined in the sediment from the clarification pond before (denoted as start sediment) and after incubation with the different carbon sources in the microcosms. When analyzing the start sediment, four different carbon substrates as well as a water control were added in triplicate to the reaction bottles. After the incubation period, PDA in sediment from each microcosm was determined both with the carbon type it had been treated with during the incubation (i.e. water, NaAc, HEC or Algae) and with the mixture of glucose, NaAc and Na- succinate.

In gas- tight 100 mL glass bottles 25 g of sediment was placed and the volume of the sediment slurry was adjusted to 30 mL by addition of distilled water. Atmosphere in the bottles was exchanged to $N_2$ by alternating between vacuum and $N_2$- gas five times. For the start sediment the analysis was performed the day after preparation, and the bottles were stored at 2°C until the analysis. The day of the analysis, the bottles were put on a rotary table (175-180 rpm) at 20 °C for 30 min to adjust to temperature before addition of the different reagents. Pure $C_2H_2$ was added to each bottle at a volume of 10 % of headspace, here 10.3 mL. Each bottle got 0.05 moles $KNO_3$ $mL^{-1}$ sediment and one of four carbon substrates or sterile water. The substrates, NaAc , HEC, mix (glucose, NaAc and Na- succinate with C in 1:1:1 ratio) and algae were added so that each bottle got 0.215 mg C $mL^{-1}$ sediment. Water was added in the same volume as the substrates. The additions were made by injecting with a syringe through butyl rubber septa in the bottle caps to ensure that anaerobic conditions were maintained. The sediments were incubated on a rotary table (175-180 rpm) and samples of 0.5 mL of headspace gas were taken in intervals of 30-40, 60, 120, 180, 240 minutes and 22 hours after the substrate injection.

The gas samples were analyzed for $N_2O$ on a gas chromatograph equipped with an electron capture detector (Clarus 500 GC, Perkin Elmer). The denitrification rate was determined as ng $N_2O$- N $g^{-1}$dw (dry weight) sediment $min^{-1}$ by non-linear regression of the $N_2O$ produced during the incubation (Pell et al., 1996).

### 3.5 REAL-TIME QUANTITATIVE PCR

DNA was extracted from the clarification pond sediment at the start and from the sediment from each microcosm at the end of the incubation. The extraction was done from 0.3-0.5 g sediment with FastDNA® Spin Kit for Soil and the FastPrep® instrument (MP Biomedicals) according to the manufacturer's instruction. Briefly, the sediment is placed in 2 mL tubes with reagents and a mixture of ceramic and silica particles. The sediment is homogenized and the microorganisms lysed with the FastPrep® instrument. The samples are centrifuged to separate the extracted DNA from sediment and reagents and are thereafter purified in several steps. The extracted DNA was quantified fluorometrically using the Qubit® platform and reagents (Life Technologies Corporation).

To evaluate the abundance of the total microorganisms and the abundance and diversity of the denitrifying organisms and organisms performing anammox, real-time quantitative polymerase chain reaction (qPCR) was used. Briefly, qPCR allows specific sequences of DNA to be amplified (copied) thousand or even a million times so that the initial number of the specific sequence can be determined (Life Technologies, 2012). In a reaction mixture containing DNA polymerase and other reagents required, the sample DNA is added together with primers which target the specific sequence of interest. By thermal cycling, alternating by warming and cooling, the specific sequence is amplified. The mixture contains a specific dye which fluoresces when bonded in a double stranded DNA. The fluorescence increases in proportion to the amount of amplified DNA and the amount of amplified sequence can be determined for each cycle. By measuring the fluorescent signal in real-time and compare to standards of known concentration, the original amount can be calculated (Life Technologies, 2012).

To determine the total bacterial community, a fragment of the 16S ribosomal RNA gene (16S) was targeted, and for the microorganisms performing anammox a taxon-specific region of 16S was used. For the denitrifying community, functional marker genes were used which encodes for specific enzymes catalyzing specific steps in in the process of denitrification. The functional markers used in this study were *nirS*, *nirK*, and *nos*ZI and *nosZ*II (Figure 2).

The qPCR reactions were performed in duplicates (four replicates for 16S and *nirS*) in a total reaction volume of 15 µL using the Biorad iQ™ SYBR® Green supermix (Bio-Rad Laboratories), 250 ng T4 pg 32 protein, 0.25-0.80 µM of each primer and 2 ng DNA. Primer sequences, concentrations and amplification protocols are found in Appendix II. By serial dilutions of linearized plasmids with cloned fragments of the specific target genes standard curves were obtained. Standard curves were linear ($R^2 > 0.997$) in the range $10^3$-$10^8$ for 16S and $10^2$-$10^7$ for the other genes. The amplification efficiency ranged between 63 % and 90 %. Non-template control reactions with only mastermix and water resulted in undetectable or negligible values. Potential inhibition of the PCR reactions was checked by comparing the amplification of a known amount of the pGEM-Teasy plasmid (Promega) with the plasmid specific T7 and SP6 primers in reactions with or without DNA extracts. No inhibition of the amplification reactions

was detected with the amount of DNA used. As an indication to verify specificity of the amplifications, melt curves were compared between samples and standards. Also a 1% agarose gel in TBE was run where the length of the amplicon determines the speed with which it moves through the gel. Samples were compared with the amplificons of standards. The size of the amplication products were as expected and no unspecific products were detected.

### 3.6 STATISTICAL ANALYZES

When replicates were used the mean of the results are presented. Standard deviation was calculated in the software Excel using STDEVP, which calculates standard deviations assuming the samples are the entire population (equation 1):

$$\sqrt{\frac{\sum(x_i-\bar{x})^2}{n}} = STDEVP \qquad (1)$$

Where $x_i$ is one of the samples, $\bar{x}$ is the sample mean and n is the sample size.

To evaluate if two sets of samples were different from each other, a two-sample Student's t-Test with unequal variance was used with an alpha value at 0.05.

When evaluating if a set of samples were different from each other, one way ANOVA with Tukey's method and confidence level of 95 % in Minitab was used. For ANOVAs, the values for PDA and the abundance of the different genes were log-transformed before analysis.

## 4. RESULTS

### 4.1 WATER AND SEDIMENT ANALYZES AT START OF THE EXPERIMENT

The result of the analyzes of the water collected from the clarification showed i.a. a total nitrogen concentration of 29.4 mg N $L^{-1}$ dominated by $NO_2^-$+$NO_3^-$, which was 28.4 mg N $L^{-1}$ (Table 4). The water content in the pond sediment was 86 % and the dry sediment had a total carbon content of 15.26% and an organic carbon content of 15.11%.

***Table 4****.Chemical parameters in the water collected in the clarification pond*

| pH | TN (mg $L^{-1}$) | $NH_4^+$-N (mg $L^{-1}$) | $NO_2^-$+$NO_3^-$ -N (mg $L^{-1}$) | $PO_4^-$-P (mg $L^{-1}$) | TOC (mg $L^{-1}$) | DOC (mg $L^{-1}$) |
|---|---|---|---|---|---|---|
| 7.4 | 29.4 | 0.714 | 28.4 | 0.028 | 2.5 | 2.3 |

### 4.2 CHEMICAL ANALYSIS OF MICROCOSMS

#### *4.2.1 SEDIMENT CHARACTERISTICS*

During the incubation, the sediments in Water and HEC did not seem to change in a way that could be observed visually (Figure 5). For NaAc, the water and sediment

developed a smell of sulphide, which was noted on the second occasion of water exchange and the smell persisted during the experiment. The fourth time of water exchange, the sediments in NaAc was black. The Algae microcosms had a layer of algae on the surface of the sediments and at the end of the experiment the top centimeter of the sediments were black.

At the end of the incubation period, the total carbon content in the sediment was only lower in NaAc compared to Start (Table 5). For the total nitrogen content, a significant decrease was observed for Water, NaAc and HEC.

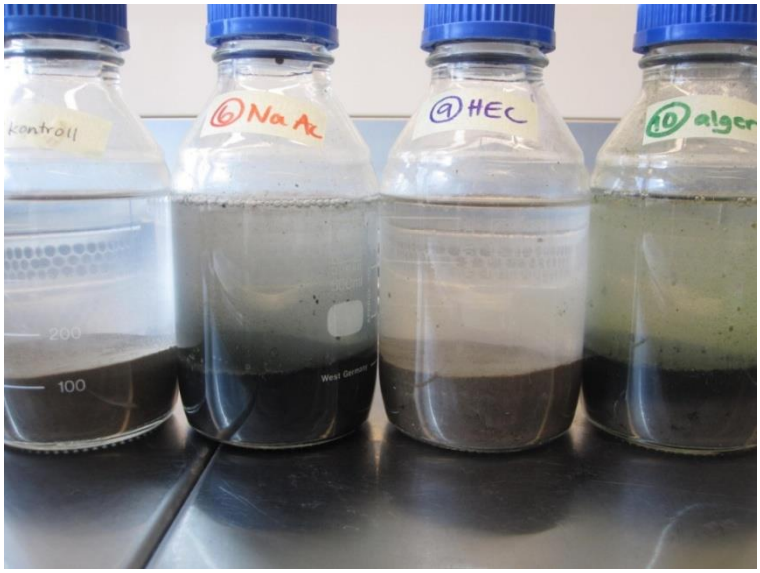


***Figure 5.*** *Microcosms (from left to right) having received water, NaAc, HEC and algae at the end of the incubation. (Photo: H. Lindberg)*

***Table 5.*** *Total carbon and nitrogen content after the incubation period) in 100 g of dry sediment (mean ±one standard deviation, n=3)*

| Sediment | TC [1](g) | TN [1](g) |
|---|---|---|
| Start | 15.26[a] (±0.39) | 0.956[a] (±0.001) |
| Water | 14.21[ab] (±0.39) | 0.895[b] (±0.005) |
| NaAc | 14.15[b] (± 0.41) | 0.882[b] (±0.001) |
| HEC | 14.52[ab] (±0.22) | 0.879[b] (±0.001) |
| Alger | 14.38[ab] (±0.15) | 0.975[a] (±0.039) |

[1]Mean values not sharing letters are significantly different ($p \leq 0.05$).

### *4.2.2 GAS PRODUCTION*

The accumulated volume of the gas produced in the microcosms was highest for Algae and lowest for Water and HEC, with NaAc in between (Figure 6).

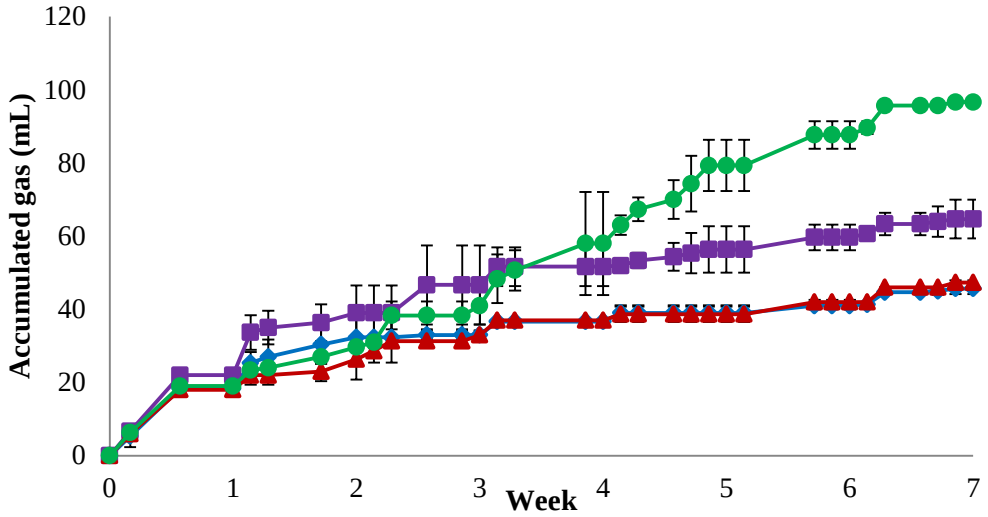


***Figure 6.*** *Accumulated gas during incubation (mean ± one standard deviation, n=3). Water (♦), NaAc (■), HEC (▲) and Algae(●).*

### *4.2.3 NITROGEN REMOVAL*

The weekly analysis of the removed water from the microcosms showed that NaAc had next to complete removal of $NO_2^-$+$NO_3^-$ every week (Figure 7). Algae had a removal of about 48-60% $NO_2^-$+$NO_3^-$ the first four weeks, after which it increased and was almost complete. Water and HEC had initially about 55 % removal, but during the experimental period it decreased to 20 and 30 % respectively. At the end of the incubation HEC had a significantly higher removal capacity than Water ($p \leq 0.05$).

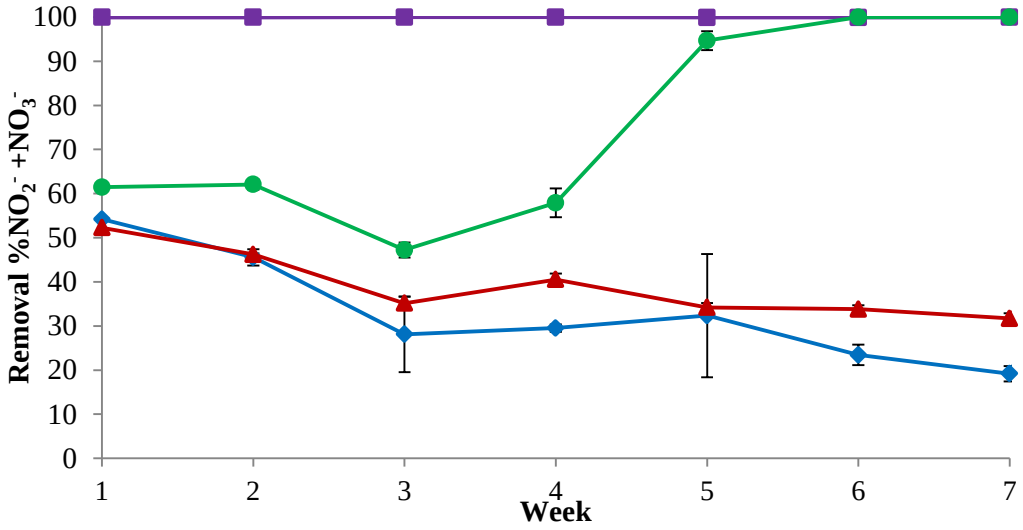


***Figure 7.*** *The percentage of $NO_2^+$+$NO_3^+$ removed during each week of incubation (mean ± one standard deviation, n=3). Water (♦), NaAc (■), HEC (▲) and Algae (●).*

The initial concentration of $NH_4^+$ in the added water each week was 0.714 mg N $L^{-1}$ (Figure 8). The concentrations after incubation with added NaAc varied between the weeks, from 0.008 to 0.8 mg N $L^{-1}$. The concentrations of $NH_4^+$ were consistently lower than the initial concentration in the Water and HEC microcosms and the value decreased during the experimental period. Algae had a higher concentration than the initial concentration in the added water almost every week. The last two weeks, the amount of $NH_4^+$ in the water from Algae increased and was about ten times higher than the initial value in the added water.

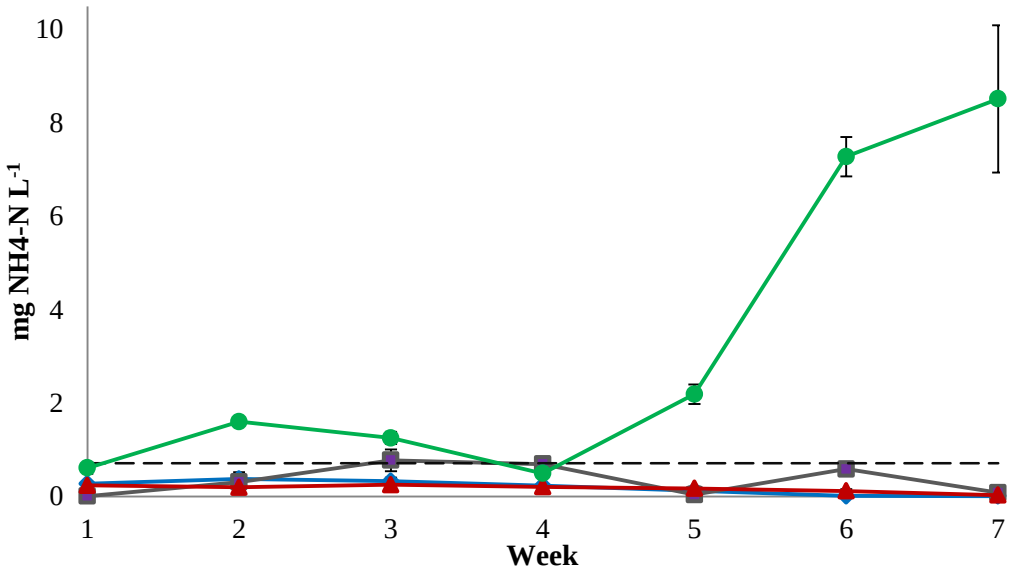


***Figure 8.*** *Concentration of $NH_4^+$ in the water in the microcosms (mean ± one standard deviation, n=3). Water (♦), NaAc(■), HEC(▲) and Algae (●), value in the added water (- -).*

### 4.3 Potential Denitrification Activity- PDA

For the Start sediment, no differences in the potential denitrification activity (PDA) was observed for any of the substrates (water, NaAc, HEC, algae and a mix of glucose, NaAc and Na- succinate) used in the assay (Figure 9). After eight weeks of incubation, the sediment from Water showed almost no activity when determined with addition of only nitrate. When instead sediment from Water was amended with C no significant difference in activity could be seen between NaAc and HEC sediments or the Start sediments. By contrast, the Algae had about four times higher denitrification rates than the other sediments, regardless of which substrate that was used in the PDA assay.

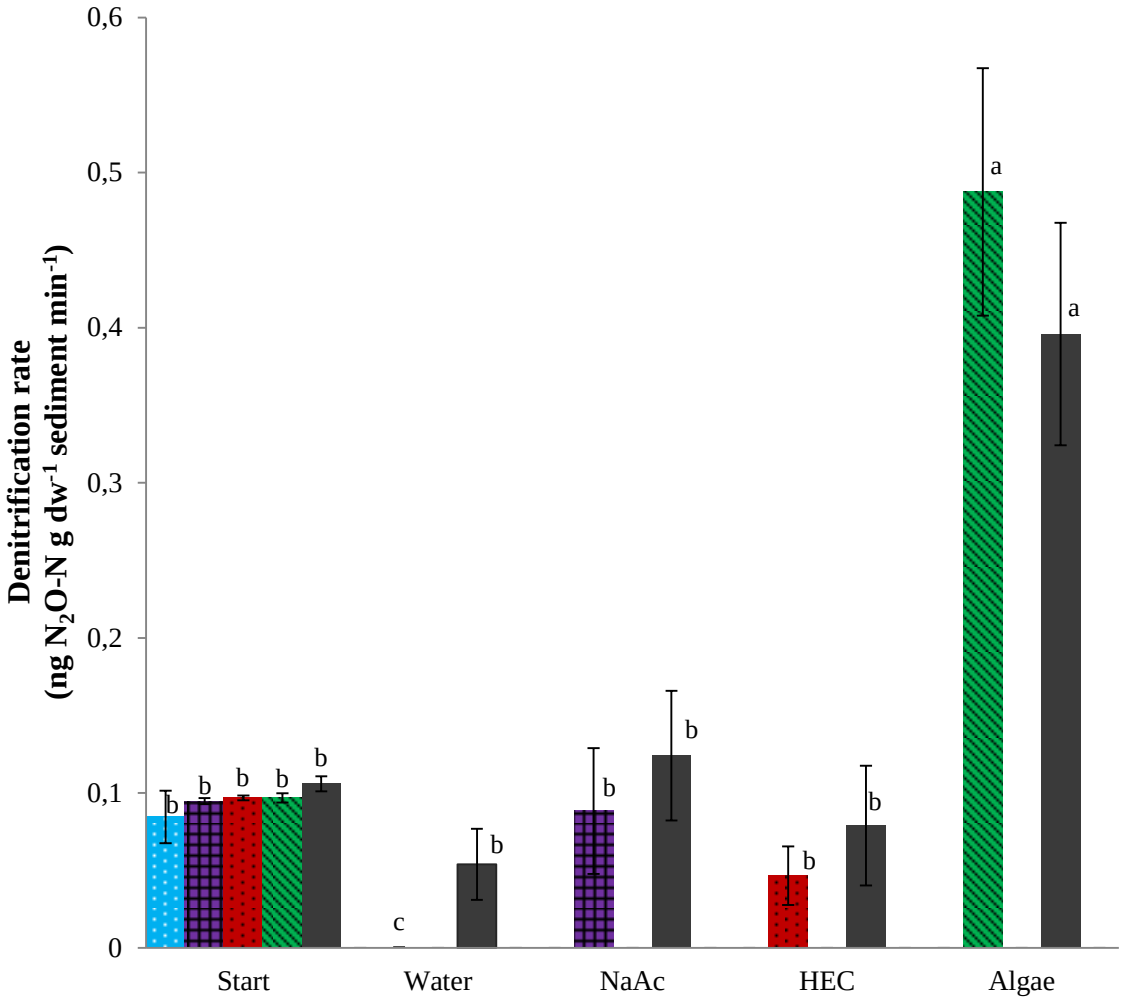


***Figure 9.*** *Potential denitrification rate in the initial sediment (Start) and in the sediment from the microcosms after incubation without carbon source (Water) or with addition of sodium acetate (NaAc), cellulose (HEC) or lysed algae (Algae; mean± one standard deviation, n=3). Additions in the PDA are denoted: water ( ), NaAc( ), HEC ( ), algae ( ) and mix ( ). Values not sharing letters are significantly different (p≤0.05).*

### 4.4 Abundance of nitrogen cycling microbial communities

When comparing the result of the microbial analyzes between the start sediment and the sediments without any addition of carbon during eight weeks, no significant difference could be seen ($p \leq 0.05$) (Table 6, Figure 10). However, the different C sources affected the abundance of some genes compared to the start sediment. Algae had a significantly larger abundance of bacteria (16S rRNA) and denitrifying community (Table 6, Figure 10). When looking at the specific denitrification genes, algae had a greater abundance of *nirS* and *nosZ*I than the start sediment. NaAc had no significant difference in 16S rRNA compared to start, however the addition had a negative impact on the abundance of the denitrifying community. The addition of HEC increased the abundances of *nirK*, *nosZ*I

and *nosZ*II compared to the start sediment. The anammox gene was more abundant in the Start, Water and HEC compared to in the NaAc.

***Table 6.****Abundance of bacterial 16S rRNA, nirS, nirK, nosZI, nosZII and anammox 16S rRNA genes (copies g dw$^{-1}$ sediment; mean± one standard deviation, n=3)*

| **Sediment** | **16S rRNA[1] x10$^{10}$** | ***nir*S[1] x10$^{10}$** | ***nir*K[1] x10$^{8}$** | ***nos*ZI[1] x10$^{8}$** | ***nos*ZII[1] x10$^{9}$** | ***Amx*[1] x10$^{8}$** |
|---|---|---|---|---|---|---|
| Start | 1.58$^{b}$(±0.23) | 0.98$^{b}$(±0.08) | 2.99$^{bc}$(±0.18) | 1.13$^{b}$(±0.11) | 1.04$^{b}$(±0.13) | 6.24$^{a}$(±0.45) |
| Water | 1.82$^{ab}$(±0.09) | 1.23$^{ab}$(±0.08) | 3.27$^{ab}$(±0.30) | 1.52$^{b}$(±0.16) | 1.59$^{ab}$(±0.13) | 6.70$^{a}$(±0.64) |
| NaAc | 1.50$^{b}$(±0.16) | 0.47$^{c}$(±0.05) | 2.17$^{c}$(±0.43) | 2.57$^{a}$(±0.36) | 0.25$^{c}$(±0.06) | 2.44$^{b}$(±0.31) |
| HEC | 1.80$^{ab}$(±0.35) | 1.21$^{ab}$(±0.10) | 4.52$^{a}$(±0.36) | 2.30$^{a}$(±0.14) | 1.72$^{a}$(±0.18) | 5.65$^{a}$ (±1.08) |
| Algae | 2.97$^{a}$(±0.82) | 1.54$^{a}$(±0.12) | 3.99$^{ab}$(±0.48) | 3.15$^{a}$(±0.47) | 1.36$^{ab}$(±0.22) | 4.21$^{ab}$(±1.03) |

[1]Mean values in a column not sharing letters are significantly different (p≤0.05).

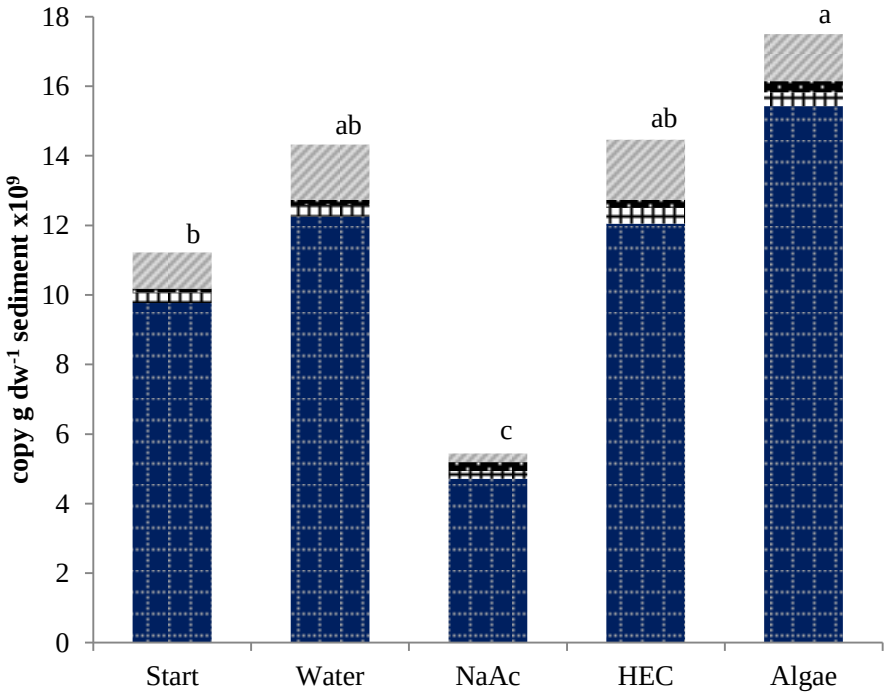


***Figure 10.*** *Abundance of the denitrifying community (entire bar), nirS ( ), nirK ( ), nosZI ( ) and nosZII ( ) per dry weight sediment for Start, Water, NaAc, HEC and Algae. Values of the abundance of denitrifying community not sharing letters are significantly different (p≤0.05).*

# 5. DISCUSSION

## 5.1 NITROGEN REMOVAL

Reduction of $NO_2^-$ and $NO_3^-$ occurred in all microcosms each week during the incubation period (Figure 7). The microcosms receiving only water had an initial removal capacity of 55 %. This was probably due to the homogenization of the sediment, which made substrates in the sediment available for the microorganisms. The relatively high temperature in which the microcosms were incubated probably also contributed, increasing the activity of the denitrifying microorganisms. During the eight weeks of incubation the removal of $NO_2^-$ and $NO_3^-$ decreased in Water, probably due to the most available substrates in the sediment being consumed.

For the microcosms with addition of HEC, which is a big and complex molecule, the results were initially similar as for microcosms amended with only water. However, the decrease in reduction during the incubation was smaller. Thus, even though HEC was a complex C source, it could increase N removal in the system. This is supported by other studies conducted in lab-scale microcosms showing that cellulose and also plant material can improve denitrification. Carbon sources like wheat straw (Ragab et al., 1992), dried plant material (Ingersoll and Baker, 1998) and a mixture of starch and cellulose (Robins et al., 2000) all enhanced denitrification. The removal in the present study was however low compared to the result from other studies, where the reduction was 88 % (Robins et al., 2000), ≥ 90% (Ingersoll and Baker, 1998) and next to complete (Ragab et al., 1992). The differences in reduction can be explained by the different types of carbon added and also by differences in temperature and incubation time (Ingersoll and Baker, 1998).

With addition of NaAc, the reduction of $NO_2^-$ and $NO_3^-$ was above 99.8% every week proving that NaAc was a simple, readily available C substrate for the denitrifying microorganisms (Figure 7). Other studies where NaAc were used to enhance denitrification also had similar results (Narkis et al., 1978; Reyes-Avila et al., 2004; Tam et al., 1992). The variations in $NH_4^+$ seen in the microcosms getting NaAc could not be explained. Since the laboratory analyzing the water is an accredited one and the handling of the samples were the same for every microcosm measuring errors were considered unlikely.

Algae resulted in a high reduction of $NO_2^-$ and $NO_3^-$ the last three weeks of incubation. The lower reduction during the first four weeks (about 60 %) could be explained by the low amount of added C week two and three, with only 12 mg C instead of the desired 49 mg (Table 2). The algae added in the beginning of the incubation could also have become more easily available for the microorganisms after some time, which also could explain the increased reduction after week four. Algae were the only C source that increased the amount of $NH_4^+$ substantially compared to the content in the added pond water. Several studies which have focused on water sediment interactions after algal blooms showed similar results as in the present study. Algae acted as a C source and enhanced denitrification and an increase of $NH_4^+$ also occurred due to remineralization

and decay of the algae (Conley and Johnstone, 1995; Philippart et al., 2000). Conley and Johnstone (1995) hypothesized that some of the $NH_4^+$ formed could be due to dissimilatory reduction of $NO_3^-$ to $NH_4^+$. This could also be the case in this study, but technical and resource limitations, made it not possible to investigate that further within the scope of this work.

### 5.2 POTENTIAL DENITRIFICATION AND ABUNDANCE OF NITROGEN REMOVING MICROORGANISMS

No significant differences could be noted in potential denitrification rates in the Start sediment regardless of addition of a C substrate or not (Figure 9). As discussed above, available substrates in the sediment could have affected the result making the additional C otiose. In the previous study of the clarification pond at the LKAB Kiruna mine, addition of C to the PDA assay increased the potential denitrification five times when assessed at15°C, compared to assays with no C addition (Ecke et al., unpublished). Two differences between the present study and the previous one are the homogenization of the sediment and the time of the year for the sampling. In the previous study, the sediment samples were collected in August 2012, whereas in the present study sediment was sampled during winter. Organic material had probably accumulated during late autumn and became available when the sediment was homogenized and kept at 20°C. The hypothesis that the available substrates in the sediment were consumed during the weeks of incubation was supported by the fact that potential denitrification rates in the sediment in Water receiving only water when determining PDA was about 100 times lower than the activity in the start sediment receiving water. Analysis of the sediment showed that the TC was higher in the start sediment compared to the sediments in all treatments after eight weeks of incubation, although the reduction in TC levels was only significant for the sediment in the NaAc- amended microcosms (Table 5).

The result from the PDA in the sediments after incubation with HEC showed that the microorganisms used HEC as a C source (Figure 9). The activity for the sediment which received HEC and which during the PDA-analysis got HEC had a significant higher activity compared to sediment from Water not receiving a carbon source. When comparing Water and HEC, it was evident that not only the results from the PDA were equal (excluding Water receiving water), but also the removal of $NO_2^-$ and $NO_3^-$ and $NH_4^+$ was and the relative abundances of the microbial genes investigated (Table 6). It was only for *nos*ZI that HEC differed and had significantly higher values compared to Water.

The abundance of the denitrifying microorganisms in sediments amended with NaAc was clearly lower compared to all other (Figure 10). The low abundance of microorganisms can explain the potential denitrifying activity for NaAc not being significantly higher than sediment from Water and HEC amended with C (Figure 9), even though NaAc had the highest reduction each week during the incubation (Figure 7). A lower abundance of denitrifying organisms likely resulted in a limitation of the active denitrifying enzymes and therefore a lower potential activity. The black sediment and the smell of sulphide ($H_2S$) indicated a reduced environment in the microcosms

(Figure 5). Sulphide is toxic to many organisms and is formed when sulphate ($SO_4^{2-}$) is reduced in an anaerobic environment by sulphate reducing bacteria (SRB) (Chen et al., 2008). The fast reduction of $NO_2^-$ and $NO_3^-$ in the presence of acetate likely lowered the redox potential allowing for reduction of $SO_4^{2-}$. If this is correct, denitrification was efficient in the microcosms amended with NaAc, although the toxic $H_2S$ inhibited the growth of the microorganisms. Another explanation to the low potential activity combined with the high removal rate is that the nitrate removal in the NaAc microcosms was supported by denitrifiers using sulphide as electron acceptor instead of C compounds (Cardoso et al., 2006).

The algae addition was the only C source resulting in significantly higher abundance of total bacteria and denitrifying microorganisms as indicated by the increase in 16S rRNA and denitrification genes when compared to the start sediment (Table 10 and Figure 10). Algae were probably a good type of addition since algae are complex and not only provide C but also other nutrients promoting growth of microorganisms. The potential denitrification activity was about four times higher for the Algae, than the activity for the other C sources which to some extent can be explained by the larger denitrifying community in Algae (Figure 10). The microorganisms in the algae treated microcosms might also have been adapted to using algae as a C source since the sediment from these had a higher activity compared to when the mix of C was added for every replicate (data not shown). However, the difference was not statistically significant due to high variation between the replicates.

The anammox specific 16S rRNA gene fragment was more abundant in Start, Water and HEC compared to NaAc (Table 5). The anammox organisms seemed to better compete in an environment with less easily available C, and this was no surprise since the microorganisms performing anammox are autotrophs. Kumar and Lin (2010) made a review of the co-existence of anammox and denitrification, and they found that during high C/N ratios denitrification and also DNRA was more competitive and that anammox was suppressed. The more abundant anammox in Water and HEC can explain the low levels of $NH_4^+$ in these microcosms (Figure 8), and this would also have reduced some $NO_2^-$.

Ecke et al. (unpublished) previously determined the abundances of the N cycling microbial communities in the clarification pond sediment at the Kiruna mine. The total abundance of the microorganisms was in the same order of magnitude compared to the present study of the clarification pond sediment and the relative abundances were similar. In both studies, *nirS* was clearly the most abundant of the determined genes, and *nosZ*II were dominant over *nosZ*I. However, the abundance of *nirS* in the previous study was about 30-40 % of the total abundance of 16S rRNA, whilst the present study was about 60 %. The same discrepancies accounts for *nosZ*I, *nosZ*II and anammox abundances, where the results in the present study are higher. As discussed, the time of the sampling probably explains differences between the studies. García- Lledó et al. (2011) who studied the microbial community in a constructed wetland found that

seasonal variations and also sediment differing in nutrient variability were the main influence of variations of the relative abundances of N-cycling genes.

### 5.3 PRACTICAL IMPLICATIONS

High levels of inorganic N in mine water effluents are problematic in many areas of the world. Methods to reduce N compounds in mine water are rare and those existing are mostly not passive (Riensel, 2001; Given and Meyer, 1998; Mattila et al., 2007; Papirio et al., 2013; Karkman et al., 2011). The use of external carbon is widespread in biological treatments where enhanced denitrification is wanted, especially when the water to be treated is low in organic material (Isaacs and Henze, 1995). For example Anoxic Biotreatment Cell (Rinsel, 2001), Fixed bed Bioreactors (Papirio et al., 2001) and Fluidized- bed Bioreactor (Karkman et al., 2011) have been used to treat mine water effluent and all used methanol as an external C source to enhance denitrification. The results of NaAc in microcosms in the present study proved that acetate is a very good C source to enhance denitrification, but to add it to the LKAB Kiruna clarification pond would not be a passive option. The addition would probably have to be done continuously and in large amounts and the solution would therefore be very costly.

Additions of C sources like cellulose and plant material, which would be a more passive treatment option, have not been investigated in ponds like the LKAB clarification pond, but have been investigated in lab-scale constructed wetlands (Robins et al., 1999; Ingersoll and Baker, 1998) and in a lab-scale anaerobic water-sediment system (Ragab et al., 1992). In all cases, these additions enhanced denitrification in the systems investigated (Robins et al., 1999; Ingersoll and Baker, 1998 Ragab et al., 1992). Plant material have potential as an external C source since it is renewable and also cheap, and the promising results from this study and others show it has potential to enhance denitrification in a passive system. However, the implementation in large scale raises questions. Will it have an effect on denitrification in a large, cold and complex system? Would the amounts of plant material needed to enhance the denitrification to a sufficient level be manageable? What happens to the water chemistry when the plant materials decay? More research is needed to be able to answer these questions.

Algae have a huge potential as a passive C source to enhance denitrification. Since algae are autotrophs the C would come from the atmosphere. One aspect of concern is the formed $NH_4^+$ during decay of algae. In a system of both oxic and anoxic zones the formed $NH_4^+$ could be decreased through nitrification in aerated zones. However, if the formed $NH_4^+$ would not be oxidized, the $NH_4^+$ could cause problems. The Swedish Environmental Protection Agency requires that LKAB does not have more than 5 µg $L^{-1}$ $NH_3$-N in the mine effluent at the Kiruna mine (Naturvårdsverket v LKAB, 2009). Considering the value of $NH_4^+$ measured in the clarification pond was 714 µg $L^{-1}$(Table 3), and is in equilibrium with $NH_3$ an increased value is not wanted. If formation of $NH_4^+$ would be at an unproblematic level, an assessment of which parameters limits the growth of the algae and the possibilities to enhance the growth would be needed. In addition, effects on recipients of the mine water would need to be considered. Since the clarification pond is not a closed system the actions to enhance algae growth in the pond

could also enhance algae growth downstream in natural lakes and rivers. Thus, instead of decreasing eutrophication it could be exacerbated. Even if algae growth could be limited to the pond, algae in the discharged water could cause problems like hypoxia in the recipients. Another matter that needs to be taken into account is the water quality of the clarification pond since the water is used in the process plants. One way to reduce problems with too much algae, both in the water recirculated to the process plant as well as to downstream recipients, is a micro-sieve in the inlet that could be installed to remove some of the algae in the water prior use (Piontek and Czyzewska, 2012).

When comparing three different constructed wetlands, Sirivedhin and Gray (2006) reported an unexpected efficiency in N removal for the youngest and least vegetative wetland. The authors proposed that the algae in a thick biofilm growing on a benthic net mesh in this particular wetland supported denitrifying bacteria with highly degradable carbon, enhancing denitrification. Other researchers have also found that periphyton (biofilm of microorganisms and organic material attached to plant roots or other surfaces) creates an environment that enhances denitrification (Toet et al., 2003; Christensen et al., 1990). Perhaps by creating an environment in the pond that promotes biofilm formation would enhance denitrification without changing the water quality and causing problems in the recipient waters.

### 5.4 Outlook

It is important to point out that this experiment was carried out at 20 °C. A lower temperature would lower the activity of the microorganisms and probably thereby also decrease the denitrification rate. Further studies should preferably be conducted at temperatures which are normally measured in the clarification pond. Further studies should also assess the possibilities to enhance the growth of algae in the clarification ponds. How an increased amount algae would affect the water quality needs to be studied as well as how and if it algal growth would affect the recipient waters. If plant material is to be considered, studies on how the decay of plants would affect the water quality and if the required amounts would be manageable are needed.

## 6. Conclusions

The potential denitrification rate in the initial sediment from the clarification pond in LKAB Kiruna mine was not immediately enhanced by addition of carbon. However, during the incubation, the removal of nitrate was enhanced by external carbon sources. The addition of algae was shown to be a good carbon source for supporting denitrification. The addition of algae increased the abundance of the denitrifying community, the potential denitrification rate increased four times after incubation and supported an almost complete removal of nitrate at the end of the incubation. The cellulose also enhanced denitrification to some extent and the abundance of the genes *nirK, nosZ*I and *nosZ*II coupled to denitrification increased. Further studies are needed to assess the practical use of external carbon sources like algae and plant material and how they would affect the complex system at Kiruna and other mining sites.

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### 7.1 PERSONAL COMMUNICATION

Emmerich, Maren, 2014. Postdoc at the Department of Microbiology, Swedish University of Agricultural Sciences.

## APPENDIX I -CALCULATION OF CARBON

The amount of C needed to reduce a certain amount of $NO_2^-$+$NO_3^-$ -N was calculated based on reaction 1:

$$5(CH_2O) + 4\,NO_3{}^- + 4H^+ \rightarrow 5\,CO_2 + 2\,N_2 + 7\,H_2O \qquad (1)$$

The amount of C needed to reduce a certain amount of $NO_2^-$ and $NO_3^-$ and being in excess was calculated using equation 1:

$$\frac{X[mg\ L^{-1}]}{14\ [g\ N\ mol^{-1}]} \cdot \frac{5[mol]}{4[mol]} \cdot 12[g\ C\ mol^{-1}] \cdot V[L] \cdot 4 = Z[mg] \qquad (1)$$

X is the concentration of N as $NO_2^-$ and $NO_3^-$, 14 is the molar mass of N, 5/4 is the ratio between the numbers of moles of C (5) needed to reduce 4 moles of N. 12 g $mol^{-1}$ is the molar mass of C. To get the C in excess the expression was multiplied with four. The amount needed in the trial was calculated using equation 1:

$$\frac{28.448[mg\ L^{-1}]}{14\ [g\ N\ mol^{-1}]} \cdot \frac{5[mol]}{4[mol]} \cdot 12[g\ C\ mol^{-1}] \cdot 0.4[L] \cdot 4 = 48.8[mg\ C]$$

28.448 is the amount of N as $NO_2^-$ and $NO_3^-$, 0.4 is the volume water and sediment added.

The amount of C in excess needed can also be described as (equation 2):

$$X[mg\ L^{-1}] \cdot V[L] \cdot 4.3 = Y[mg\ C] \qquad (2)$$

X is the concentration of N as $NO_2^-$ and $NO_3^-$, V is the volume sediment and water and 4.3 is the constants in equation 1.

50 mL solutions of NaAc and HEC were prepared so that 1 mL of the solution reduces 10 mg N $L^{-1}$. Using equation 2, where X is 10 mg N $L^{-1}$ and the volume is 1 mL the amount of carbon needed in each mL solution is 43 mg C $mL^{-1}$. Following equation describes how the amount of NaAc or HEC was prepared (equation 3):

$$\frac{43\ [mg\ C\ mL^{-1}] \cdot 50[mL] \cdot Mw[g\ mol^{-1}]}{Mw_C\ [g\ C\ mol^{-1}]} = Amount\ of\ substrate\ to\ 50\ mL\ water\ [mg] \qquad (3)$$

Mw is the molecular weight of the substrate and $Mw_C$ is the molecular weight of the carbon in the substrate. The result of equation 5 is the amount of substrate (mg) to add to 50 mL water.

NaAc is a salt with sodium (Na) and acetic acid ($CH_3COOH$) with the total formula $C_2H_3NaO_2$. A solution of NaAc was prepared according to equation 5 with Mw as 136.08 since 3 water molecules ($H_2O$) is attached as well. $Mw_C$ is 24 and this will give the amount of NaAc as 12.2 g to 50 mL water.

HEC is a big molecule with a molecular weight of about 90 000 g $mol^{-1}$. The fraction of carbon is 120/228 which with equation 5 give 4,085 g HEC to 50 mL water.

Carbon content in the algae was determined for the first four additions with optical density (OD) as a function of carbon content in algae. OD was measured using "CO8000 Cell Density Meter WPA biowave" in a 10 step dilutions series of 8 mL and the carbon content was determined in each of the dilutions (figure 13).

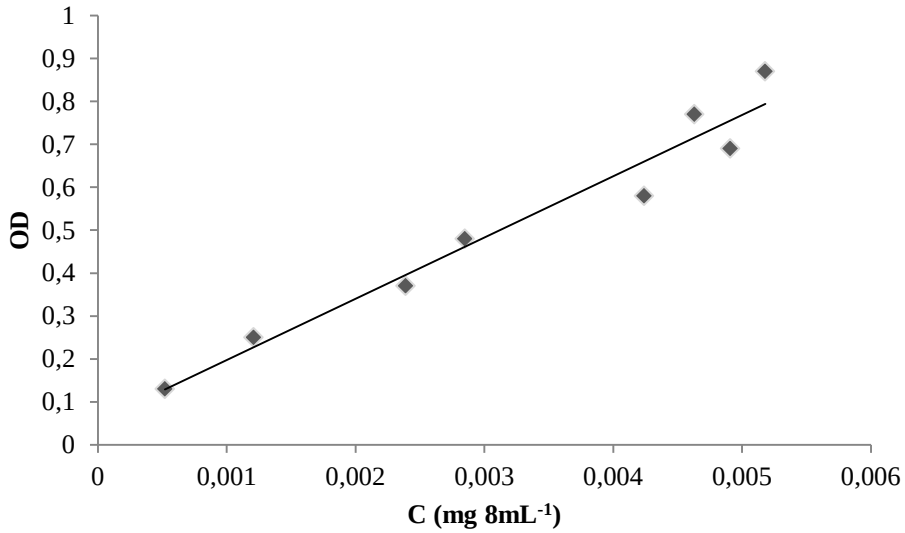


***Figure 11.*** *OD on y-axis agains carbon content in 8 mL of algae dilution of different concentrations.*

The eqation describing the relationship between carbon content and OD in 8 mL solution can be seen in equation (equation 4):

$$y = 142.72x + 0.0549 \quad (4)$$

When OD is equal to 1 the C content (x in equation 4) is 0.8 mg C $mL^{-1}$ of OD 1. To calculate the C content in one mL algae solution by OD the following equation was used:

$$C = 0.8 \cdot OD \quad (5)$$

## APPENDIX II -PCR PRIMERS AND THERMAL CYCLING CONDITIONS USED FOR QUANTIFICATION OF DIFFERENT GENES

| Target gene and primers | Sequence (5'-3') | Primer conc. (µM) | Thermal cycling conditions (flouresent signal acquired at 80 or 70 °C in all protocols) |
|---|---|---|---|
| ***16S rRNA*[1]*:*** | | 0.5 | (95°C, 5 min) x 1<br>(95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 78°C, 10 s) x 35<br>(95°C, 15 s;(60 to 95° C, 5 s, increment 0.5°)), x 1 |
| 341F | CCT ACG GGA GGC AGC AG | | |
| 534R | ATT ACC GCG GCT GCT GGC A | | |
| ***nirK*[2]**:<br>nirK 876<br>nirK R3Cu | ATY GGC GGV CAY GGC GA<br>GCC TCG ATC AGG TTR TGG TT | 0.25 | (95°C, 5 min) x 1<br>(95°C, 15 s; (63°C – 58°C, -1°/cycle), 30 s; 72°C, 30 s) x 6;<br>(95°C, 15 s; 58°C, 30 s; 72°C, 30 s; 80°C, 10 s) x 35<br>(95°C, 15 s;(60 to 95° C, 5 s, increment 0.5°)), x 1 |
| ***nirS*[3]**:<br>nirSCd3aFm<br>nirSR3cdm | AAC GYS AAG GAR ACS GG<br>GAS TTC GGR TGS GTC TTS AYG AA | 0.8 | (95°C, 5 min) x 1<br>(95°C, 15 s; (65°C – 60°C, -1°/cycle), 30 s; 72°C, 30 s) x 6;<br>(95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 80°C, 10 s) x 35<br>(95°C, 15 s;(60 to 95° C, 5 s, increment 0.5°)), x 1 |
| ***nosZI*[4]**:<br>nosZ2F<br>nosZ2R | CGC RAC GGC AAS AAG GTS MSS GT<br>CAK RTG CAK SGC RTG GCA GAA | 0.5 | (95°C, 5 min) x 1<br>(95°C, 15 s; (65°C – 60°C, -1°/cycle), 30 s; 72°C, 30 s) x 6;<br>(95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 80°C, 10 s) x 35<br>(95°C, 15 s;(60 to 95° C, 5 s, increment 0.5°)), x 1 |
| ***nosZII*[5]**:<br>nosZII F<br>nosZII R | CTIGGICCIYTKCAYAC<br>GCIGARCARAAITCBGTRC | 0.8 | (95°C, 5 min) x 1<br>(95°C, 15 s; 54°C, 30 s; 72°C, 30 s; 80°C, 30 s) x 40<br>(95°C, 15 s;(60 to 95° C, 5 s, increment 0.5°)), x 1 |
| **Amx 16S[6] (anammox)**<br>AMX 818 F<br>AMX 1066 R | ATG GGC ACT MRG TAG AGG GGT TT<br>AAC GTC TCA CGA CAC GAG CTG | 0.8 | (95°C 5 min)x1 (95°C 15s, 60°C 30 s, 72°C 30 s, 80°C 10 s)x 40;<br>(65 to 95 °C, 10 s, increment 0.5°C)x1 |

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