The University of South Bohemia in České Budějovice
Faculty of Science

Using molecular and culturing methods to study nitrifying microorganisms in peat

Bachelor thesis

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Annotation

Soil samples from two peatland sites were analysed in order to determine the population of nitrifying microorganisms. Two DNA extraction methods were compared according to yield and an enrichment culture was used to attempt to isolate ammonia-oxidising organisms in a pure culture.

Key words:

peatland soil, nitrification, bacteria, archaea, DNA extraction, 16S rRNA, culture, fen, spruce mire

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 15th December 2022 .....

Eliška Daňková

Acknowledgments

I would first like to thank Anne Daebeler for accepting me into her lab and her team for the duration of this thesis. Secondly, I would like to thank Vojtěch Tláskal and Justus Nweze for their support, guidance and undying patience in and outside of the lab. Thank you for holding me accountable and cheering me on. Lastly, but not in any way less, I would like to thank my partner, Bernhard Glocker, for keeping me fed and alive during the time of my studies.
Thank you all for being there for me and for being just awesome people.

Abstract

Nitrification, one of the key components of the global nitrogen cycle and the organisms performing its steps in peatlands influence nutrient availability. Many nitrifying organisms have not yet been cultured nor studied; therefore, the goal of this thesis was to quantify the abundance of nitrifying organisms in two sites and to isolate them in pure cultures. A further question was posed; to find the more effective way to isolate DNA from peat soil samples. Soil cores (3 per site in 2 layers) were taken from a fen and a spruce mire site in Šumava National Park, the pH of the resulting 12 samples was determined. Microbial DNA was extracted from the soil samples using two methods, sequenced for the 16S amplicon and analysed. The efficiency of the methods was compared by the acquired DNA concentration. The spruce mire peat soil was more acidic than the fen soil and from the two extraction methods, the phenol/chloroform method had a significantly higher yield. Nitrifying activity was detected in two steps of enrichment. Two genera of nitrifying organisms were found in the sites; Nitrobacter (NOB) and Nitrosospira (AOB) in fen and spruce mire respectively. The pH of each site is correlated to its vegetation cover and water table. The choice of extraction method should depend on time constraints and exact goal – purity or quantity. The presence of nitrifying organisms of a site has shown to depend on the site properties (pH, nutrients) and on the aeration of the soil layers. The potential for the process of nitrification in these sites was proven, which may provide the basis for further research on the ecology of nitrifiers in these systems. The successful steps resulting in an enrichment culture could lead to establishment of a new culturing method for acidophilic nitrifying organisms and a new acidophilic ammonia-oxidising culture.

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1. Introduction

1.1 Nitrification

One of the key components of the global nitrogen cycle was found to be nitrification, which is the conversion of ammonia to nitrite and subsequently to nitrate (Ward, 2011). A crucial role in nitrate leaching and ground water pollution is connected to this conversion. On the other hand, it is being used in waste water treatment plants to convert ammonia/ammonium to nitrate and thereby to fuel the further conversion of N–compounds via denitrification to nitrogen gas. This process serves to prevent water pollution and eutrophication.

1.1.1 Ammonia Oxidation, Nitrite Oxidation, Complete Ammonia Oxidation

Nitrification is carried out in two steps – the ammonia oxidation and nitrite oxidation. Those can either be carried out by two different types of microorganisms or one type of microorganism.

Microorganisms that use ammonia as their primary source of energy and carry out the first step of nitrification are called ammonia oxidising microorganisms. In the process of ammonia oxidation only a very limited amount of energy can be produced (complete nitrification: $\Delta G^{\circ\prime}$ = - 349 kJ mol^{-1} NH_3, ammonia oxidation to nitrite: $\Delta G^{\circ\prime}$ = - 275 kJ mol^{-1} NH_3, nitrite oxidation to nitrate: $\Delta G^{\circ\prime}$ = - 74 kJ mol^{-1} NO_2^-; Daims *et al.*, 2015) and therefore the growth of ammonia oxidisers is slow. The ability to use ammonia oxidation was found in both bacteria (AOB) and archaea (AOA; Bollmann *et al.*, 2011; Winogradsky, 1890; Winogradsky, 1892; Treusch *et al.*, 2005; Venter *et al.*, 2004).

The second step of nitrification – nitrite oxidation to nitrate; is facilitated by nitrite–oxidising bacteria (NOB), for example from the genus *Nitrobacter*.

Until the discovery of complete ammonia oxidisers (comammox) in the genus *Nitrospira*, it

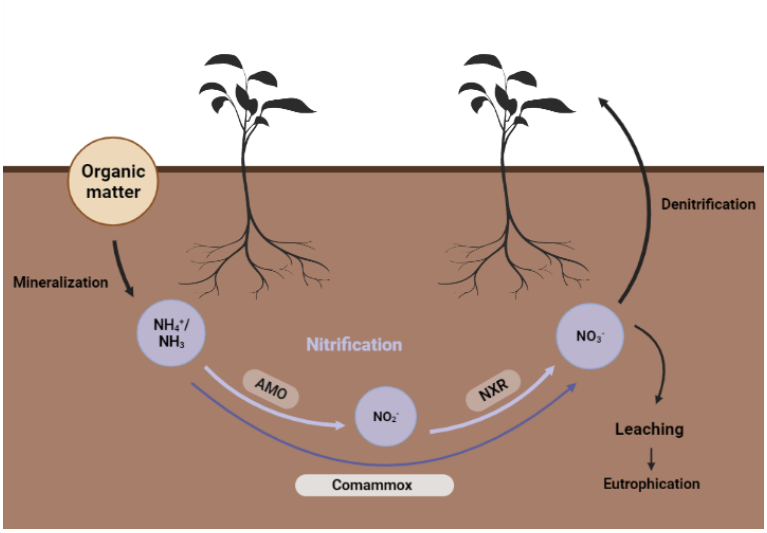

Figure 1.1: Simplified scheme of nitrification featuring the corresponding genes and the organisms, by which both nitrification steps are performed; the ammonia oxidising archaea (AOA) and ammonia oxidising bacteria perform the first step, the nitrite oxidising bacteria (NOB) that perform the second and commamox organisms that perform both steps. Ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR) are the enzymes that facilitate the corresponding step of nitrification. Figure was created using BioRender.com.

was thought that one organism cannot perform both steps of nitrification (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Comammox metabolism was found in many oxic environments including a varied community in soils (Koch *et al.*, 2019) and waste water treatment plants and seems to possess an ammonia monooxygenase (AMO) that is phylogenetically distinct from AMOs in AOA and AOB (Daims *et al.*, 2015; van Kessel *et al.*, 2015).

1.1.2 Factors Affecting Nitrification in Acidic Environments

At low pH the ammonia availability is reduced by ionisation, which is believed to limit the growth of ammonia–oxidising archaea and bacteria growing in liquid media culture (Lehtovirta-Morley *et al.*, 2011). The same organisms may be, in the absence of nitrite oxidation, inhibited by an elevated concentration of both HNO_2 and HNO_3 and the NO and NO_2 generated from HNO_2. These oxides have shown to be unstable and reactive at low pH (Lehtovirta-Morley *et al.*, 2011). However, nitrifying organisms have been detected and isolated from acidic soils (Lehtovirta-Morley *et al.*, 2014, Lehtovirta-Morley *et al.*, 2011).

1.2 Commonly Used (Molecular) Identification Methods for Nitrifiers

There are many levels on which the microbial organisms can be studied, for example: the phenotype, genomics and transcriptomics – the latter of which are molecular methods. The phenotype is used to determine the shape and colour classification but not taxonomy, as that has shown to be insufficient. The phenotype is also used to describe the growth and replication rate, and certain environment–specific coping mechanisms, for example spore formation.In genetic analysis the study of DNA is generally used for taxonomic purposes, for the annotation of genes and identification of mutations within them. Transcriptomics is used to study the expression of genes; genes which have been actively translated to ribosomal RNA and mRNA. As the focus of this thesis was set in the microbiological field and only molecular methods were used, this text will be further focused solely on those.

Commonly in environmental microbiology, groups of bacteria and archaea are identified by their 16S rRNA gene, which contains species–specific hyper–variable regions, is targeted with primers, amplified, sequenced and analysed. Polymerase chain reaction (PCR) is usually used for the gene amplification, followed by one of many sequencing methods; frequently Sanger or Illumina sequencing is used. Sequences are then assigned to taxonomic groups and a phylogenetic tree can be constructed to give the final taxonomic affiliation of the microorganism hosting the particular 16S rRNA gene. If the focus is placed on a specific group of microbes, primers for genes specific to the group have to be designed and used. For example, to differentiate between the AOA, AOB and comammox organisms, the gene for subunit A of ammonia monooxygenase (amoA) specific to the group is targeted (Pjevac *et al.*, 2017). Since the phylogeny of the amoA gene follows that of the 16S rRNA gene well (Aakra *et al.*, 2001) this analysis results in a robust identification.If only the quantification of a group of organisms is desired, the quantitative PCR method is used.

The studied microorganisms can be characterised using either culturing or non–culturing methods. With the culturing method, a pure culture is obtained first, usually from an enrichment culture, before the microorganism is genomically analysed (Dunfield *et al.*, 2010; Auman *et al.*, 2000). The pure culture can also be used for metabolic analyses of the organism, for enzyme kinetics characterisation, and more. For the non–culturing method, DNA is extracted

directly from an environmental sample. It can then be further used for deep taxonomic surveys of microbial community structures or the prediction of functional potential of the whole microbiome in the sample. With the DNA–based approach, it is possible to study microorganisms which have not yet been successfully cultured and therefore cannot be studied by culturing–based methods, for example some anaerobic archaeal species (Vaksmaa *et al.*, 2017).

The selection of the DNA extraction method is a crucial step in the workflow of the DNA–based approach as it directly influences the amount and quality of the DNA, available for downstream analyses (Alteio *et al.*, 2021). Furthermore, each experiment usually requires optimization of the amount of sample input into the selected extraction method and selection of the necessary clean–up steps to further purify the extracted DNA.

1.3 Peat as a Niche for Microorganisms

There are several types of peatlands with distinct properties defined and protected by the Ramsar convention (Gardner and Davidson, 2011). Three main ecological niches can be found within peatlands; elevated hummocks that are relatively dry and therefore covered with plants which need more support from soil, intermediate lawns that are wetter and usually covered with smaller plants and wet hollows that are covered mostly with *Sphagnum* mosses. Wet hollows are the most important for the peatland's function as a carbon sink, as *Sphagnum* biomass is resistant to microbial decomposition (Chroňáková *et al.*, 2019).

The peat types that were focused on in this thesis are denoted as a fen and a spruce mire. A mire is a wetland fed by ground water and atmospheric precipitation (*i.e.* minerotrophic and ombrotrophic, respectively), contrary to a fen, which is fed by ground and surface water (Chroňáková *et al.*, 2019). Further differences include nutrient availability, acidity and vegetation; fens are defined as the nutrient rich and with higher pH compared to mires, their vegetation cover is comprised mostly of vascular plants (like grass–like graminoids), whereas the vegetation cover of mires is also comprised of woody plants (shrubs and trees). In the latter, the growth of woody plants is enabled by a lower water table present in the sites. All of these site characteristics together with internal niches within the sites shape the composition of the unique microbial community (Chroňáková *et al.*, 2019, Urbanová and Bárta, 2014).

In general, fens are characterised by a higher relative abundance of *Actinobacteria*, *Proteobac-*

teria and *Verucomicrobia*, whereas spruce mires have been found to be characterised by a higher abundance of *Acidobacteria* (Urbanová and Bárta, 2014, Urbanová and Bárta, 2016). A few trends have been described regarding the preference of microbial taxa for environmental conditions: Most phyla, except for *Acidobacteria*, were found to show increased relative abundance in correlation with rising pH. At the same time, they were found to be negatively correlated with total C and N content (Urbanová and Bárta, 2014).

2. Aims

- To compare the yield of two DNA extraction methods for acidic peat soil – phenol/chloroform extraction and extraction using a commercial kit (MP Bio FastDNA Spin Kit for Soil).
- To identify the abundance and taxonomic identity of peat nitrifiers by sequencing of the 16S rRNA gene.
- To enrich an ammonia–oxidising microorganism culture.

3. Materials and Methods

3.1 Collection of Peat Soil Samples and Analysis of Their Basic Properties

The peat soil samples were collected on the 16th of October 2021 at two locations in the first zone of the Šumava National Park in the vicinity of Kvilda, Czech Republic. One site was a spruce mire (49.0173572N, 13.5673000E) and the other a fen (49.0166411N, 13.5685650E). The spruce mire was covered by *Picea abies* and the herbal vegetation was dominated by blueberry and cottongrass (*Vaccinium sp.* and *Eriophorum sp.*, Fig. 3.1a). The water table at this site was 15 cm below the surface level. The fen site was chosen to represent an open type of peatland with dense grass vegetation dominated by *Carex rostrata* and a high water table (5 cm below the surface, Fig. 3.1b).

From each site, 3 soil cores were taken by a squared metal soil corer (10×10cm). The living *Sphagnum* part was removed and each soil core was divided into an upper (0 – 10 cm) and a lower (11 – 30 cm) layer. The upper layer consisted mainly of dead *Sphagnum* biomass and the lower layer consisted of a dark soil matrix with high content of organic matter . This resulted in twelve samples in total.

3.1.1 pH Analysis

From each sample, approximately 2 – 3 ml of water were placed into a 15 ml falcon tube and shaken at 4°C for 2 h before the pH was measured with an inoLab® pH 720 pH meter probe (Thermo Fisher, Germany). Each measurement was conducted carefully in such a way that the glass probe would not touch the walls of the tube, which could hinder the accuracy of the measurement.

(a) Spruce mire site

(b) Fen site

Figure 3.1: A visual representation of the sites and their vegetation cover. (a) spruce mire, covered by *Picea abies* and herbal vegetation dominated by blueberry and cottongrass (*Vaccinium sp.* and *Eriophorum sp.*). (b) An open type of peatland is represented by the fen site, with dense grass vegetation dominated by *Carex rostrata*. Both photos were taken by Vojtěch Tláskal and Justus Nweze.

3.2 DNA Analysis of Peat Soil Samples

The analysis of the deoxyribonucleic acid (DNA) of all samples was performed to assess and compare the microbial community of each site.

3.2.1 DNA Extraction

For the extraction of DNA, two methods were used and their yields were compared. These were: a) the FastDNA^{TM} SPIN kit for soil (MP Bio, USA) as the commercially available and potentially safer option and b) a standardized phenol–chloroform extraction protocol (Angel *et al.*, 2021).

The Commercial Kit Extraction

In order to extract DNA by a commercially available kit, the FastDNA^{TM} SPIN kit for soil was used (MP Bio, USA). The selection of the kit was made on the basis of previous satisfactory yields of DNA from other experiments in our laboratory. The corresponding protocol provided by the manufacturer was used to perform the extraction as follows.

A fraction of each sample was weighed to be ca. 350 mg (wet mass) and placed in the Lysing Matrix E tube (MP Bio, USA) with abrasive pellets. Two buffers were used to chemically disrupt the cell walls of the target organisms, solubilize other possible contaminants present in the soil samples and later stabilize the DNA. MP FastPrep–24^{TM} 5^{G} (MP Bio, USA) (40", 6.0 m/s) was used to mechanically disrupt target cells and therefore release DNA into the solution. The samples were then spun in the 5430 R Eppendorf centrifuge (Merck KGaA, Germany) (14 000×g, 5 min) to pellet all insoluble particles. The supernatant was pipetted into a 2 ml microcentrifuge tube and the Protein Precipitation Solution (MP Bio, USA) was added to help precipitate all proteins in the samples. The precipitate was then pelleted by centrifugation (14 000×g, 5 min) and the supernatant was transferred to a SPIN^{TM} filter (MP Bio, USA) containing a silica matrix for DNA binding. The filter was washed with the SEWS-M solution (MP Bio, USA) diluted with ethanol to remove impurities, and subsequently dried to remove the ethanol. Finally, the DNA was eluted in 50 µl of DNase/Pyrogen-Free Water (DES) and stored at -20 °C for further use.

The Phenol-Chloroform Extraction

To extract DNA without the use of a commercial kit, the protocol optimized by Angel et al., (2021) was followed. Briefly, ca. 350 mg (wet mass) of each sample were weighed in a Lysing Matrix E tube (MP Bio, USA) containing glass beads. The sample was chemically lysed by a mixture of buffers and phenol and mechanically by shaking in the MP FastPrep–24^{TM} 5^{G} (MP Bio, USA) (30", 6.5 m/s). This was repeated two more times to ensure complete lysis. Then, phenol (P) / chloroform (C) / isoamyl alcohol (IAA) (25:24:1) was added following centrifugation (14000 rpm, 15°C, 3 min) to precipitate proteins from the samples. A second protein precipitation was carried out with the supernatant by addition of a mixture of C / IAA (24:1). Subsequently, the DNA was precipitated using RNA–grade glycogen and PEG Precipitation Solution (60 g PEG (M.W 7000-9000), 18.7 g NaCl, 200 ml RNase–free water) and centrifuged (14000 rpm, 4°C, 60 min) to form a pellet. This pellet was then washed with ice–cold 75% ethanol which was then removed again, and the pellet was air dried at room temperature. Lastly, the pellet was resuspended in 100 µl of Low–EDTA TE buffer (500 µl Tris–HCl (1 M, pH 8.0, Trizma), 10 µl EDTA (0.5 M, pH 8.0), 50 ml RNase–free water) and the resulting DNA samples were stored at -20 °C for further use.

Inhibitor Removal

To ensure the precision of DNA concentration measurements and the success of PCR amplification, the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA) was used to remove humic substances from samples (Angel *et al.*, 2021). The DNA extract was centrifuged through 2 columns with rewetted resin (due to high sample coloration) and placed back at -20 ° C.

3.2.2 DNA Concentration Measurement

Two DNA quantification methods were used. A NanoDrop One instrument (Thermo Fisher Scientific, USA) was used as an easy and fast option to verify the success of our extraction. The PicoGreen method was then used to compare the yield of the two extraction methods used. The following section describes both methods in more detail.

DNA Concentration Measurement with NanoDrop®

The concentration of DNA extracted with the FastDNA™ SPIN kit for soil (MP Bio, USA) was measured with a NanoDrop One instrument (Thermo Fisher Scientific, USA) to verify the success of our extraction and its approximate purity. A second extraction using the FastDNA™ SPIN kit was then performed from all samples to provide a representative DNA sample from the soil and to increase the total extracted mass of DNA, which was needed for further steps of the study.

DNA Concentration Measurement with PicoGreen®

For additional DNA measurement the Quant–IT™PicoGreen® dsDNA Assay Kit (ThermoFisher, Germany) was used to quantify DNA was used according to the manufacturers' instructions. Five DNA concentration standards (200 ng/µl, 100 ng/µl, 20 ng/µl, 1 ng/µl, 0 ng/µl) were filled in duplicate in plate wells and the DNA samples were diluted 100–fold with 1× TE buffer(1.1 ml of 20×TE buffer, 20.9 ml of nuclease–free water). Then 1 µl of the DNA samples was mixed with 99 µl of 1× TE buffer and pipetted into the remaining empty wells. Subsequently, all filled wells were supplemented with previously prepared PicoGreen® work solution and incubated in the dark (room temperature, 2 min). The fluorescence of the

standards and samples was then measured with a Synergy HTX Multimode Reader (BioTek, USA) with an excitation of ca. 480 nm and at an emission of ca. 520 nm.

3.2.3 PCR

The 16S rRNA prokaryotic gene in DNA samples acquired by phenol/chloroform extraction was amplified using barcoded primers 515F and 806R by three PCR reactions per sample (Caporaso *et al.*, 2012). The PCR premix contained 2.5 µl 10× Green Taq Buffer (Thermo Fisher Scientific, USA), 2.5 µl dNTP set, 0.1 µl BSA, 0.625 µl of each primer, 0.125 µl Green Taq polymerase (Thermo Fisher Scientific, USA), 17.525 µl PCR water and 1 µl of undiluted template DNA. The PCR conditions for amplification were 5 min at 94°C, 25 cycles (45 s at 94°C, 45 s at 52°C and 45 s 72°C) and 10 min at 72°C. The amplified samples were then visualized by agarose gel electrophoresis (1% agarose) to confirm a successful amplification. Subsequently, the samples were sent for sequencing to UIC Sequencing Core, Chicago, USA by 2×150 bp Illumina sequencing.

3.3 Enrichment of an Ammonia Oxidiser from Peat Soil

40 ml of enrichment medium containing 400 µl of minimal mineral salt medium, 34.48 ml of autoclaved water from the sites, 400 µl 10× KH_2PO_4 (KH_2PO_4 170 mM , K_2HPO_4 720 mM), 40 µl trace element solution, 4 ml 10× $CaCO_3$, 100 µl antibiotics (50 mg/L), 40 µl 1 mM NH_4Cl, 40 µl SeW solution, 500 µl BTB solution (0.01% in ultra pure water) was prepared into 100 ml glass bottles. All components of the medium were autoclaved separately. It was decided to use no buffer after no growth was detected when using $CaCO_3$, instead the pH was monitored and corrected as needed. A fraction of each soil sample was placed in the prepared medium for enrichment. The ammonia oxidizing activity was detected with Quantofix NO_2^-/NO_3^- strips (MACHEREY–NAGEL, Germany) and sulfamic acid (100 mM), which converts nitrite to di–nitrogen and thus allows to detect presence of nitrite and nitrate in the medium with the strips. The presence of ammonia was checked using the Nessler's reagent coloration (Yuen and Pollard, 1954). After sufficient activity was detected, 2 ml of the original culture were transferred into new medium without antibiotics in an effort to purify the culture.

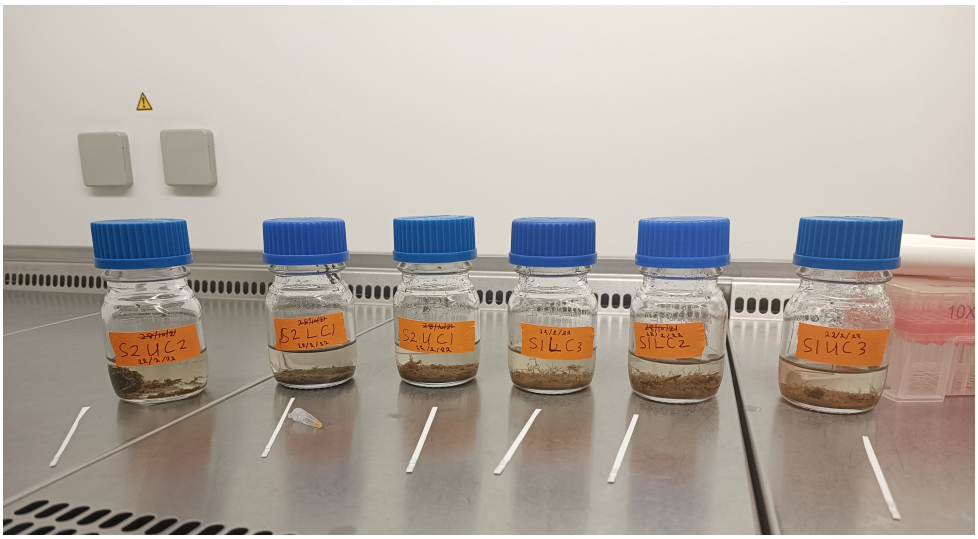

Figure 3.2: A photo of the set–up of the first step of the culturing process, including the paper strips and Nessler's reagent used to check the progress of ammonia oxidation in the cultures. The fractions of the soil samples used for enrichment can be seen at the bottom of the bottles.

3.4 Sequence Analysis

After the sequence data was received from the sequencing company, the data was processed and analysed. Cutadapt was used to remove all adapters, barcodes and primers (Martin, 2011). The dada2 package (Callahan *et al.*, 2016) from R was used to de–noise and remove sequencing errors from the data from the received paired–end fasta files. The read quality profiles were inspected and the sequences were filtered and trimmed accordingly. The error rates were assessed and the sample inference was calculated. The paired reads were merged and the amplicon sequence variant table (ASV) was constructed. Taxonomy was then assigned to this table, using the naive Bayesian classifier method (Wang *et al.*, 2007), from two databases (SILVA r132; Quast *et al.*, 2012 and GTDB; Parks *et al.*, 2022) and the resulting tables were combined. This taxonomic table was then used for further statistical analysis in R (R Core Team, 2021). The absolute 16S rRNA sequence counts were transformed into relative values for better comparability of presence of taxonomic groups.

3.5 Statistical Analysis

The data was analysed using R Studio with R v.4.2.1. A Welch Two Sample t–test and ANOVA adjusted with Tukey Honest Significant Differences (TukeyHSD) were used to test

for significant differences in pH between sites. A two–way ANOVA was further used to test for significant interactive effects of site and soil layer with pH. A paired t–test was used to test for significant differences in DNA yield between samples obtained by the two extraction methods used. For each of the detected ASVs the mean relative abundance of three replicate samples at the phylum and genus level was calculated. After testing the data distribution with the Shapiro–Wilk normality test and finding a normal distribution, an ANOVA was used to identify significant differences of selected relative abundances of genus–level ASVs between soil layers and sites. A non–metric multidimensional scaling analysis (NMDS) based on relative abundance of the prokaryotic ASVs from the dominant phyla and Bray–Curtis distance of the samples was made to identify differences in microbial community composition between all samples and to detect possible similarities of samples coming from the same site.

To make graphs, the ggplot package from the tidyverse collection was used to help visualise the data in barplots and boxplots (Wickham *et al.*, 2019).

4. Results

4.1 pH Analysis

The mean values of pH at each site were 4.52 ± 0.17 and 3.89 ± 0.25 for fen and spruce mire, respectively (4.1). This difference in mean pH was found to be significant (Welch Two Sample t–test: P = 0.00076). The distribution of pH values in the samples was shown to not be layer–dependent but to only be dependent on the site (two–way ANOVA P = 0.40, P = 0.00098, respectively).

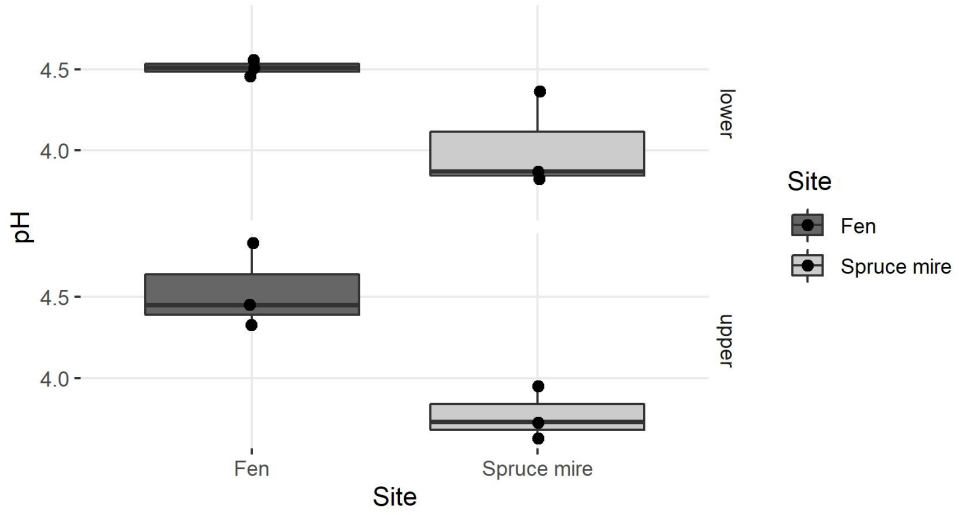

Figure 4.1: Variation of pH in the peat samples in lower and upper layers of fen and spruce mire sites. Each dot represents one sample. The median across the replicates (n=3) is indicated by the horizontal line of the boxplot. The spread shows the standard deviation.

4.2 DNA Extraction

Two extraction methods were used and compared for all samples. The phenol–chloroform extraction method was shown to be significantly more effective than the FastDNA^{TM} SPIN kit

for soil extraction (paired t–test: p–value = 1.859e-05, Fig. 4.2). Using the extraction with the kit, more pure; less colored, therefore containing less humic acids, samples were obtained.

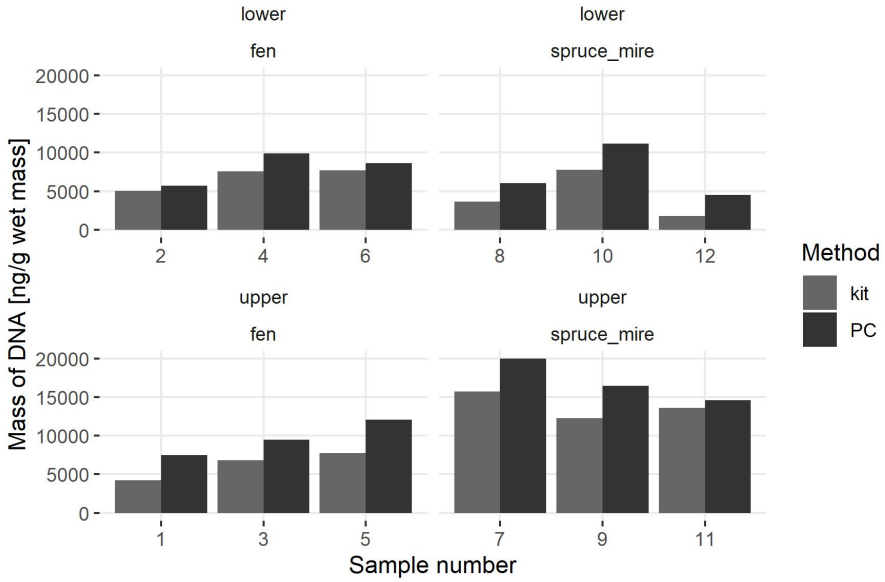

Figure 4.2: Yield of DNA after extraction from fen and spruce mire peat soil samples of the upper and lower layers.

4.3 Enrichment of an Ammonia Oxidiser from Peat Soil

Low activity as determined by very faint coloration of the nitrite/ nitrate indicator stripes (Fig. 4.3), was observed in the enrichment within 6 weeks. After a transfer to fresh medium and another 5 weeks of incubation still only faint activity was detected.

4.4 Sequence Analysis

From the total number of detected phyla, seven most abundant were chosen to analyse and displayed (Fig. 4.4).). These seven phyla contained approximately 60 – 80% of the total diversity an there was no apparent difference in phylum–level diversity between the layers within the sites and between the sites themselves. *Proteobacteria* (*Alphaproteobacteria*, *Gammaproteobacteria*, mean relative abundance = 33.4%) and *Acidobacteria* (mean relative abundance = 22.9%) were the most abundant phyla in both sites. The phyla were chosen according to Urbanová and Bárta, (2016) , including the following five, which contributed significantly less to the overall

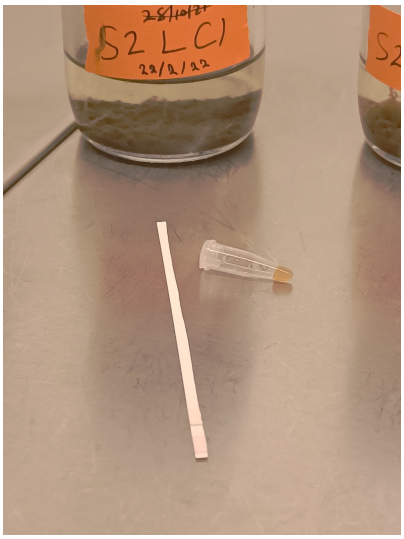

Figure 4.3: Exemplary representation of the results on the nitrite/nitrate test strip. The faint pink coloration of upper field on the strip shows the presence of nitrate. The yellow coloration of Nesslers' reagent in the test tube is indicative of the presence of ammonium.

relative abundance: *Verrucomicrobiota* (7.8%), *Actinobacteriota* (5%), *Bacteroidota* (4.3%), *Chloroflexota* (1.3%) and *Elusimicrobiota* (0.3%).

Since the focus of this thesis was placed on nitrifiers, the relative abundance of the detected nitrifying organisms was plotted. Only two genera were detected: *Nitrobacter* (a NOB) and *Nitrosospira* (an AOB), both of which had low relative abundance (Fig. 4.6). *Nitrosospira* (0.02%) was only detected in the spruce mire site, exhibiting a higher relative abundance in the lower layer (ANOVA: P = 0.052). *Nitrobacter* (0.06%) was detected in both layers of the fen site and in one sample of the spruce mire site. ASVs of this genus were more abundant in the upper layer than in the lower layer of the fen site (ANOVA: P = 0.349).

A significant difference in microbial community structure was found between the two peat soil sites (Fig. 4.7). Samples from the spruce mire site had a greater difference between layers than the fen samples (Fig. 4.7).

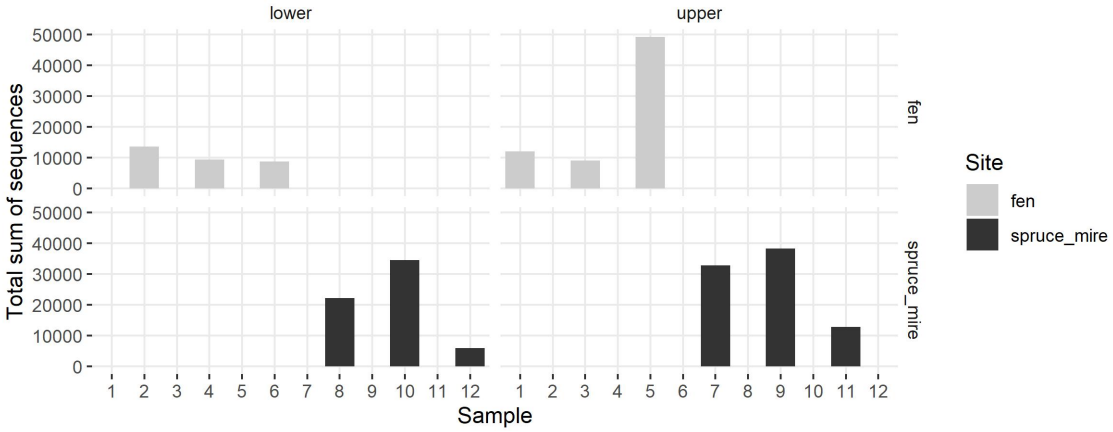

Figure 4.4: Number of sequences (sequence counts) in individual samples, from the fen and the spruce mire sites. Results for samples from the upper and the lower peat soil layers are shown.

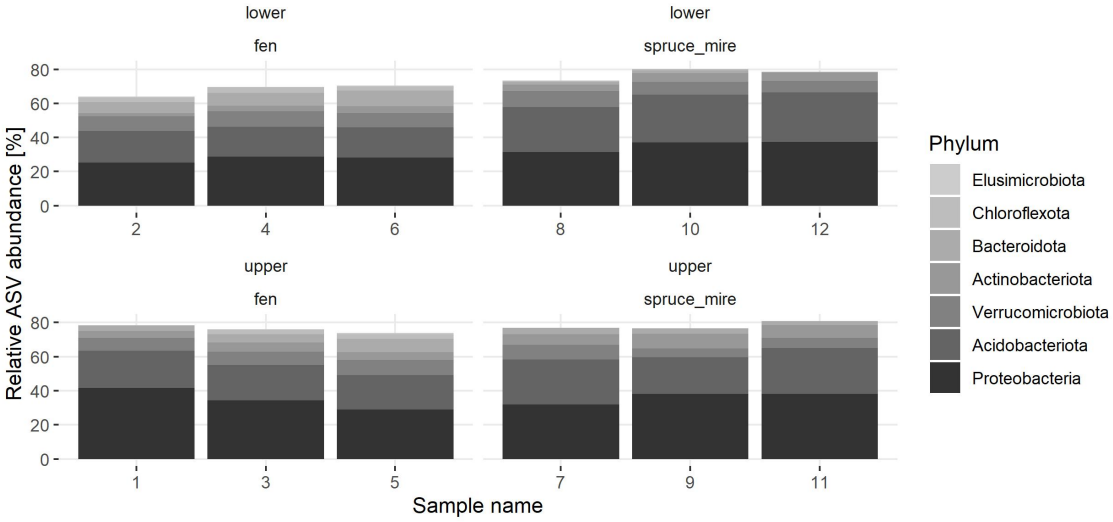

Figure 4.5: Relative abundances of ASVs from the most abundant phyla detected in samples from the fen and spruce mire sites collected from the upper and lower soil layers. The top abundant phyla and those found to be most relevant in Urbanová and Bárta, (2016) were chosen for display.

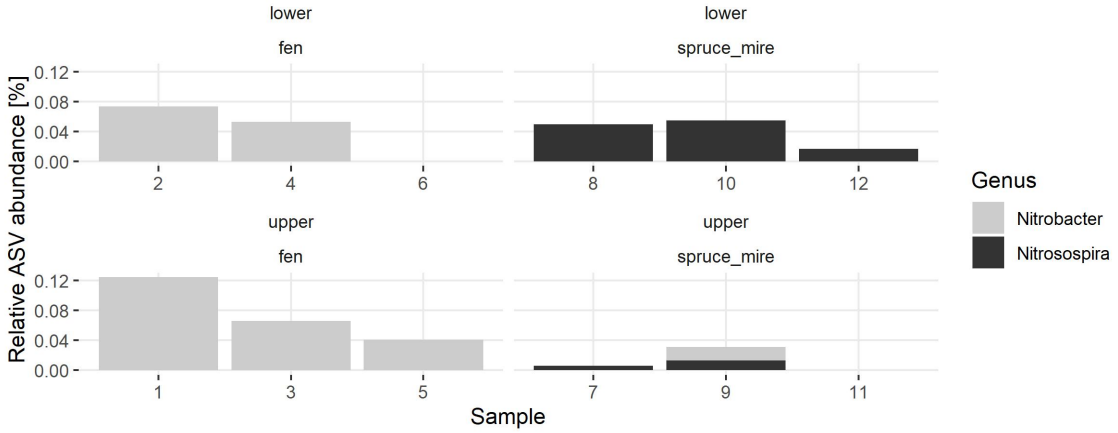

Figure 4.6: Relative abundance of the two detected genera of nitrifiers in the peatland soil samples from the fen and spruce mire sites collected from the upper and lower soil layers.

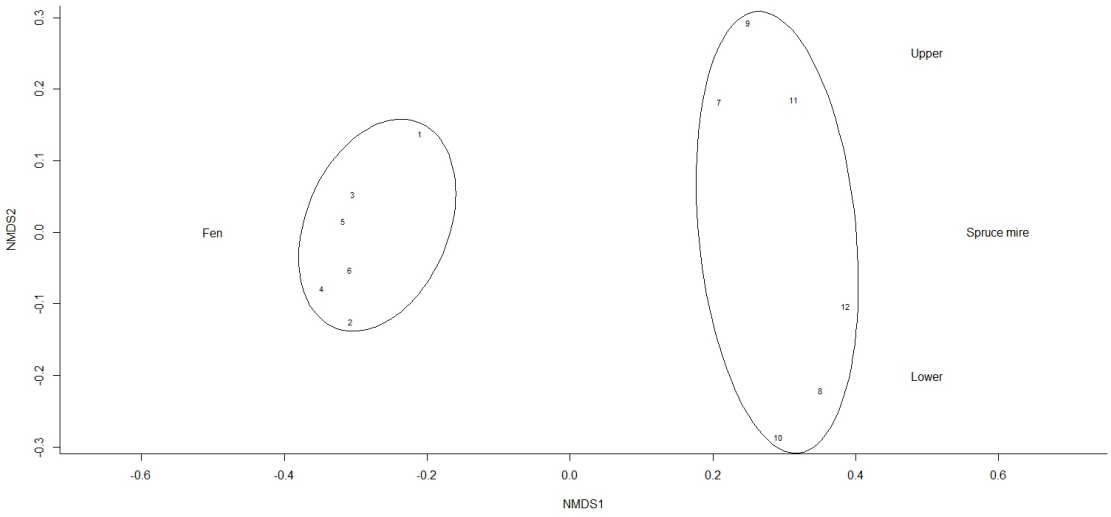

Figure 4.7: Biplot of an NMDS analysis of the 16S rRNA amplicon–based prokaryotic communities from the seven most relevant phyla (see Fig. 4.5). The ellipses show 95% confidence intervals around groupings of communities from the fen and spruce mire sites. The stress value is 0.0245.

5. Discussion

5.1 pH Analysis

The samples from the spruce mire site had a significantly more acid pH than the samples from the fen site. This difference in pH is likely connected to the different vegetation cover which in turn is affected by the difference in the overall water regime (Chroňáková *et al.*, 2019, Urbanová and Bárta, 2014). Acidification of soil in spruce mires has been shown to be caused by high input of litter from coniferous spruce trees and its decomposition in these sites (Chroňáková *et al.*, 2019). Litter decomposition is favored in this peat type since large amounts of water are transported into the atmosphere through the tree tissues by evapotranspiration and in turn the water table is lowered, allowing for aerobic decomposition. The decomposition of organic litter by microorganisms and the resulting production of soluble molecules which lower pH m are stimulated by such lower water content in the soil (Chroňáková *et al.*, 2019).

5.2 DNA Extraction

Two methods were used for the extraction of DNA from soil peat samples. A few differences in the results obtained by the two methods were observed: The kit method provided more visually clear samples while the phenol–chloroform method gave slightly more coloured samples containing more DNA. An increased concentration of humic acids that inhibit PCR and interfere with DNA concentration measurement, could be colorationthe reason of the darker color in the phenol–chloroform extracted samples. The difference in yield of extracted DNA was found to be at the significantly in favour of the phenol/chloroform extraction and it can therefore be concluded that the extraction efficiency of this method is higher for the peat soil of the studied sites.

5.3 Enrichment of an Ammonia Oxidiser from Peat Soil

The enrichment culture was transferred to fresh medium once but no further due to low activity and time constraints. The absence of a buffer in the medium made it difficult to keep a steady pH in the cultures and could have negatively affected the growth of the target organisms, which may prefer acidic pH conditions. The very low starting abundance of the target organisms and them being slow growing microorganisms could be the reasons for the overall low detected activity in both transfers of the cultures. The target organisms are aerobic and prefer stationary growth conditions, therefore keeping them in stationary closed bottles could mean that all oxygen is quickly depleted and the organisms are then inhibited until the culture is opened again (Kits *et al.*, 2017). Also the possible absence of supporting partner organisms could have impeded the growth after transfer (Lehtovirta-Morley *et al.*, 2011). Such partners are often vital for detoxification of the media from metabolic products of ammonia–oxidisers such as nitrite (Lehtovirta-Morley *et al.*, 2011).

5.4 Sequence Analysis

The relative abundance of the top two detected phyla (*Proteobacteria* and *Acidobacteria*) was correlated negatively with pH, which, in the case of *Proteobacteria* is contradictory to previous findings (Urbanová and Bárta, 2014, Urbanová and Bárta, 2016). The differing relative abundance of the other five more closely inspected phyla (*Verrucomicrobiota*, *Actinobacteriota*, *Bacteroidota*, *Chloroflexota* and *Elusimicrobiota*) cannot be explained from the available data but is probably connected with the vegetation cover and pH (Chroňáková *et al.*, 2019). The presence of two different genera of nitrifiers (*Nitrobacter* and *Nitrosospira*) was detected within the sites. *Nitrobacter*, nitrite–oxidizing genus present in the fen, was found to display up to 0.12% of the total prokaryotic relative abundance in the upper layer of this habitat. Its presence also in the lower layer could be connected to aeration of this layer by long roots of *Carex sp.*. The *Nitrosospira*, ammonia–oxidizing bacterial genus present in the spruce mire, seems to prefer the lower layer with larger water saturation, which could be correlated with the better nutrient availability or aeration by spruce roots (Chroňáková *et al.*, 2019). Oxic layers of the soil or micro–patches to which oxygen is diffused through root tissues are most likely preferred by both nitrifiers. Low abundance of nitrifiers caused by low energy yield and long doubling

time is in line with findings from other habitats such as waste water treatment plants (Lücker *et al.*, 2015) or other freshwater habitats (Sauder *et al.*, 2011, Sauder *et al.*, 2018). In general, activity of nitrifiers in pristine peatlands without anthropogenic pollution is constrained by strong limitation of available nitrogen. The majority of available ammonia, the key substrate for the first step in nitrification, is probably assimilated by other organisms into amino acids. Despite nitrogen limitation in pristine peatlands (Urbanová and Bárta, 2014), performance of nitrification is suggested by detection of nitrifiers in the soil of the sites studied by this study.

6. Conclusion

The first goal of this thesis was to compare the yields of DNA extraction from peatland soil samples using FastDNA^{TM} SPIN kit for soil and phenol–chloroform extraction. The extraction using FastDNA^{TM} SPIN kit for soil provided lower yield but cleaner samples. The phenol–chloroform extraction had higher yield but required an additional clean–up step. Thus, the use of each method would be advised according to time constraints – kit or preference for higher extraction efficiency – phenol–chloroform.

The second goal was to identify nitrifying microorganisms in the soil samples and to examine their relative abundance. The presence of two distinct nitrifying genera were detected within the peatland soil samples using the 16S rRNA gene; *Nitrobacter* (NOB) and *Nitrosospira* (AOB) in fen and spruce mire respectively. A site specific distribution of those genera was observed together with a layer specific distribution that can be correlated to the oxygen availability in said layer. This data compared to the abundance of methanothrophic microorganisms could reveal interesting relationships within the groups and reveal further relationships with their environment.

The third goal was to enrich a nitrifying culture. This endeavour was successful, although the culture was slow–growing to a point, which is beyond the time–frame of this thesis, therefore a pure culture was not acquired. With further experiments, this method could lead to the establishment of a new culturing protocol for acidophilic nitrifying microorganisms and a new acidophilic ammonia–oxidising culture.

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