Master's Thesis

Phenanthrene biodegradation potential and diversity of aspen rhizosphere bacteria

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Harri Kovakoski: *Phenanthrene biodegradation potential and diversity of aspen rhizosphere bacteria*
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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous, often carcinogenic and persistent soil pollutants that disperse over long distances. In phytoremediation plants are used to support soil microbiota capable of degrading pollutants. I studied the effects of soil pH and glucose supplement on the degradation of phenanthrene (PHE) in soil samples collected from a phytoremediation site consisting of a plantation of different aspen species. The site at Somerharju, Luumäki, was originally contaminated with PAH-compounds during creosote impregnation, and the phytoremediation experiment was started by Finnish Natural Resources Centre (LUKE) in 2013. The soil respiration of radiolabelled PHE was studied with the Microresp™ device in a two-week incubation experiment. Microbial content of soil samples' was sequenced using next generation sequencing (NGS) of the 16S rRNA gene to explore the differences in the microbiota diversity of the rhizosphere. Both aspen species increased PHE degradation of the rhizosphere soil samples when compared to controls, but no difference was observed between the plots planted with European aspen or hybrid aspen. Soil pH and the addition of glucose did not significantly increase degradation of PHE. Local diversity was largest in soil samples collected from plots without trees, while the plots with trees had more between-samples variation. The results suggest possibilities for the use of the key species *P. tremula* and targeted bacteria strains in remediation.

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Polysykliset aromaattiset hiilivedyt (PAH) ovat kaikkialle maailmaan levinnyt joukko usein karsinogeenisiä ja hitaasti hajoavia maaperän saasteita. Fytoremediaatio on maaperänpuhdistusmetodi, jossa kasveja käytetään tukemaan maaperän saasteita hajottavia mikrobeja. Tässä pro gradu-tutkielmassa tutkin maaperän pH:n ja glukoosilisän vaikutusta fenantreenin (PHE) hajoamiseen maanäytteissä, jotka oli kerätty Suomen Luonnonvarakeskuksen (LUKE) hallinnoimalta fytoremediaatio-alueelta Somerharjulta, Luumäeltä, jossa kasvoi eri haapalajeja. Kunnostettava alue on puretun kreosoottikyllästämön saastuttama ja Suomen Luonnonvarakeskus (LUKE) on aloittanut fytoremediaation vuonna 2013. Radioleimatun PHE:n hajoamista tutkittiin MicrorespTM-laitteella kaksi viikkoa kestävässä kokeessa. Maanäytteiden metagenomi sekvensointiin seuraavan sukupolven geenisekvensointimenetelmillä (NGS) näytteiden mikrobien diversiteetin selvittämiseksi. Sekä metsähaavat että hybridihaavat lisäsivät PHE hajotusta tilastollisesti merkittävästi kontrolleihin verrattuna, mutta ei toisiinsa verrattuna. pH:lla ja glukoosilla ei ollut tilastollisesti merkittävää vaikutusta hajotukseen. Mikrobien paikallinen diversiteetti oli korkeimmillaan kontrolleissa, mutta puita sisältävissä näytteissä oli enemmän näytteiden välistä variointia. Tulokset tarjoavat mahdollisuuksia avainlaji *P. tremulan* ja kohdennettujen bakteerilajien käyttöön puhdistuksessa.

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TERMS AND ABBREVIATIONS

TERMS

Rhizosphere	"The zone of soil around a plant root, in which the root exerts an influence on the growth and distribution of microorganisms" (Pearce et al., 1997)
Soil priming	Induced increase in soil derived carbon or nitrogen release by the means of adding substances such as glucose into soil (Kuzyakov, Friedel and Stahr, 2000)

ABBREVIATIONS

Clone 134	*Populus tremula* x *Populus tremuloides*
Clone R4	*Populus tremula*
CPM	counts per minute
HC	hydrocarbons
LUKE	Finnish Natural Resources Centre
NGS	next generation sequencing
OTU	operational taxonomic unit
PAH	polycyclic aromatic hydrocarbons
PHE	phenanthrene
rRNA	ribosomal RNA

1 INTRODUCTION

A major need arisen during the past decades in environmental protection is remediation of soils contaminated with toxic compounds. One major group of such soil pollutants are polycyclic aromatic hydrocarbons (PAHs). PAHs are a family of chemicals, which contain more than one benzene ring (Figure 1; Tong et al., 2018).

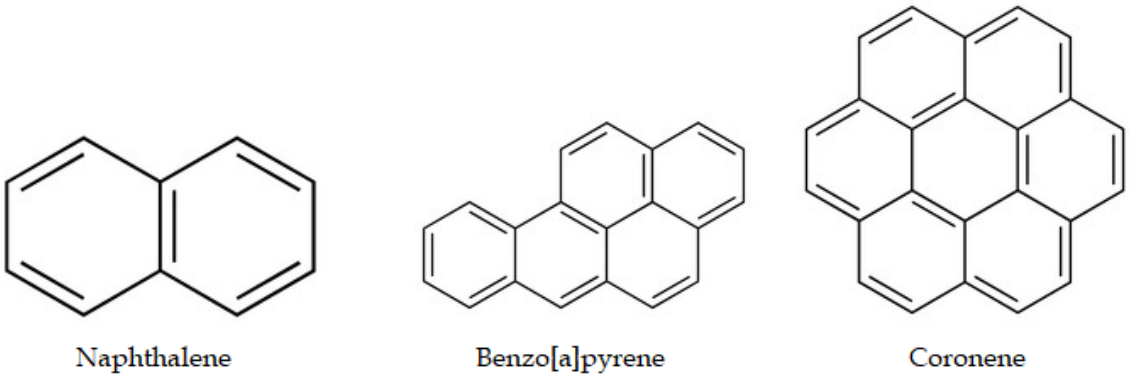


Figure 1. Structural formula of 3 different PAHs, each of which contains more than one aromatic ring (Royal society of chemistry, 2020)

PAHs are carcinogens (IARC, 1983) the toxicity of which varies according to their structure (Weis et al., 1998). Due to their chemical structure they are also persistent organic pollutants (POPs) i.e. substances that persist long in the environment and are able to biomagnify and bioaccumulate in the environment (WHO, 2020) which further increases their harmfulness. For example, in Shanghai the amount of PAHs in the soil can exceed acceptable threshold concentrations increasing the risk of cancer for local farmers (Tong et al., 2018).

Zhang and Tao (2004) estimated that the production of 16 different PAHs in the year 2004 was 520 Gg. Of these emissions, combustion of biofuel was globally the greatest PAH source, contributing 56,7 % of all emissions, and wildfires were the second greatest source, contributing 17,0 %. The contribution of different sources differs spatially, e.g. in the USA the greatest contributor to PAH emissions is

consumer product usage (35,1 %) calculated as per capita emissions, followed by traffic oil (23,0 %). PAH sources can also vary seasonally, as was demonstrated in the study of Han et al. (2020), where biomass and coal combustion were identified as the major PAH sources in China during the winter time and oil and gas activities during the summer. PAHs are also created through natural processes, which can even be the dominant source of PAHs in some regions, but globally and in many regions, anthropogenic sources outweigh natural ones (Zhang and Tao, 2004).

PAHs are also capable of long distance atmospheric transportation and can travel far from their source of origin, as was demonstrated by Klanova et al. (2008) who postulated that the urban areas of Africa, South America and Australia are the sources of PAHs found in the soils of Antarctica. Considering this along with an estimated number of 36 000 – 55 000 PAH-contaminated manufactured gas plant sites in the USA alone (Kuppusamy et al., 2017) it is easy to understand why PAH contaminated soils are considered a ubiquitous problem.

Considering the dangers PAHs pose and the possible interest to utilize the polluted sites, there is a great need to remediate sites contaminated with PAHs. A plethora of different remediation techniques is available for soil remediation including and not limited to stabilization, incineration, soil vapor extraction and bioremediation (Kuppusamy et al., 2017). These techniques vary in their efficiency in removing PAHs from contaminated soils, e.g. 24 % of PAHs were removed during 8 weeks of electrokinetic treatment and 35 % total PAH were removed during 16 h *in vitro* fatty acid methyl ester washing (Isosaari et al., 2007; Gong et al., 2010). The cost of these remediation techniques also vary: $40 to $60 per cubic yard using stabilization/solidification, $2 to $450 per cubic yard using soil vapor extraction and $10 to $60 dollars per cubic yard using bioremediation processes (US EPA, 2000).

One possible method for soil remediation is phytoremediation i.e. cleaning pollutants by growing plants in the polluted soil. Plants are known to accumulate soil pollutants directly to their biomass (Dhiman et al., 2016) and are also proven to

stimulate biodegradation performed by microbes living in their rhizosphere (Ying et al., 2011). The rhizosphere can be defined as "the zone of soil around a plant root, in which the root exerts an influence on the growth and distribution of microorganisms" (Pearce et al., 1997). The bacteria in the rhizosphere have the ability to mineralize hydrocarbon (HC) type pollutants (Pui-Yi et al., 2001) and degrade them by using them as a carbon source in cellular respiration (Boldrin, Tiehm and Fritzsche, 1993; Rabodonirina et al., 2019).

Plants support pollutant degrading bacteria in their rhizosphere in many ways. Plants activate pollutant degradation abilities in bacteria (Toussaint et al., 2012), improve degradation conditions in the soil e.g. by improving oxygen availability (Singer et al., 2009) and excrete substances that work as co-metabolites in PAH degradation (Rentz, Alvarez and Shnoor, 2005). Research has indicated that the amount of bacteria and pollutant degraders is higher in plant rhizosphere compared to bulk soil (Nie et al., 2009). Rhizosphere microbiota is also reported to have a more diverse gene pool compared to bulk soil (Li et al., 2014). The symbiotic relationship in pollutant degradation benefits the host plant as well. The bacteria in the rhizosphere are known to reduce the stress plants experience due to toxic compounds e.g. by inhibiting the production of stress ethylene (Burd, Dixon and Glick, 1998).

Plenty of research exists on the efficiency of different plant species in PAH phytoremediation, but research specifically with aspens is scant or has provided negative results. For example, a previous study showed that the presence of hybrid aspens (*Populus tremula* × *tremuloides l. P.* × *Wettsteinii*) did not increase the degradation of either total oil substances or PAHs over a four year *in situ* experiment (Sillanpää, 2007). Research on the differences in phytoremediation efficiency between different aspen variants is lacking as well.

In this study, I sought to compare the potential of the rhizosphere microbes of two aspen variants, European aspen (*Populus tremula*) and hybrid aspen (*Populus tremula*

x *tremuloides*), to soil bacteria with no rhizosphere effect to metabolize a single PAH**.** To the best of my knowledge, no research on the use of European aspen (*Populus tremula*) in phytoremediation of PAHs has been reported (Pulkkinen, 2019). To the best of my knowledge no comparison of the remediation efficiencies of aspens or hybrids has been published either. Several possible influencers on degradation besides aspens were identified and researched in the same study. Firstly, the experimental setting was designed so that the effect of soil pH on degradation could also be examined. The experimental setting also allowed a relatively uncomplicated research on the effects of added substrates on soil carbon respiration, a method known as soil priming (Kuzyakov, Friedel and Stahr, 2000). This study sought to determine whether glucose would increase the degradation of a chosen pollutant. Lastly, environmental metagenetic methods were used to give insights into active bacteria present in the samples and the mechanics behind bacterial degradation in the samples. In this study I used next generation sequencing (NGS) to describe the differences in the microbial communities with or without a rhizosphere effect.

The aim of the study was to assess if the potential of pollutant degradation, measured as the amount of CO_2 respiration of ^{14}C labelled pollutant was significantly different

1) in soil samples with different pH values

2) between samples with and without added glucose

3) between samples with and without trees

4) between samples with different tree species in their vicinity.

Additionally, it was studied if samples taken from the same sampling plot produced similar amounts of labelled CO_2.

The hypotheses related to this study are:

H_1) soil pH can affect the potential for PHE degradation

H_2) glucose addition can prime the PHE degradation in soil and rhizosphere

H_3) aspen trees affect the potential of PHE degradation of the rhizosphere soil compared to samples with no aspens

H_4) rhizospheres of different aspen tree types (European aspen and hybrid) have differences in their PHE degradation potentials

H_5) there are differences in bacterial diversity between samples with different aspens and samples with no aspens

The H_0 hypothesis is that none of the treatments have an effect on PHE degradation or have differences compared to one another in terms of PHE degradation and tree species, and that treatments do not affect the bacterial diversity.

2 MATERIALS AND METHODS

Samples were collected from Somerharju, Luumäki (Figure 2, coordinates ca. 60.915591 N, 27.423932 E (Suomenkartta, 2019) from a site with an ongoing phytoremediation project governed by The Natural Resources Institute of Finland (LUKE). A creosote impregnation facility has operated on the site until the 1950s and the soil of the 1,5 hectare area is polluted with several PAHs and other organic pollutants (Metla, 2018a; Metla, 2018b; Golder associates, unpublished). The site is now owned by Senate Properties (Senaatti-kiinteistöt) after property rights passed to them from Finnish Transport Infrastructure Agency (Ratahallintokeskus) in 2008.

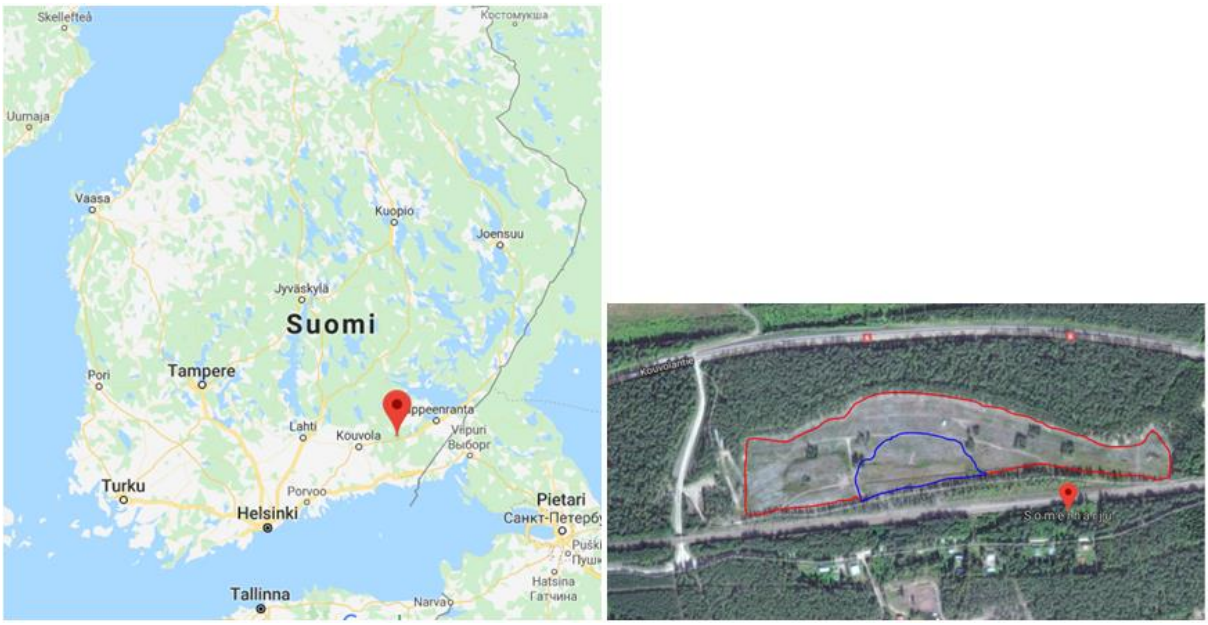


Figure 2. Left: Somerharju located on map, right: outliers of Somerharju restoration site. The outer line in red on the right-hand picture indicates the border of the polluted site and the inner blue line the rough location of the most intently polluted area. Google maps, from the Internet 22.11.2019

The restoration site is an even area between an esker and a railway bank with a cleared sand road running through the site. The vegetation consists mostly of planted aspen trees along with lichens and some moss and heather. Whilst visiting the site in 2018 the soil conditions were very dry after a hot summer with little rain.

The site contains aspens of different species in a very dense culture (around 10 000 plants per hectare) that were planted in the area by LUKE with the aim to remediate the soil. The project is the first of its kind in Finland, as these kinds of phytoremediation processes have so far only been carried out in laboratories, whereas the test in Somerharju will be the first full-scale long term phytoremediation process. The process started in 2013 and has been running for 7 years (Pulkkinen, 2019).

2.1 Sample collection

The most intently polluted area on the Somerharju site was divided to equal sized squares with a diameter of couple dozen meters, each of which was planted with one of the 10 different aspen species, in 2013. The area was divided to 4 replicates of squares of each of the 10 tree species. A sand road runs through the area. Squares of two aspen types were selected to be sampled for this study and are here called sample plots.

To compare PHE respiration with and without rhizosphere effect, samples were collected either close to living trees or removed from trees (the control samples) either by collecting them from the sand road running through the area with no trees or from areas within the plots where trees had died through natural reasons. For each sample one 1 litre sealable plastic bag of topsoil and one bag of deep soil from each sampling spot was collected to be combined later. The word sample from here on refers to top and bottom soil combined as one. Sample combination is explained further in section 2.2.1.

48 samples in total were collected. Firstly, 16 samples were taken from the sample plots of European aspen (*P. tremula,* origin: Loppi) named the "R4" species by LUKE. Secondly 16 samples were taken close to hybrid aspen trees (*P. tremula* x *P. tremuloides*) a.k.a. the "134" species. Finally, 16 control samples were taken either

from sampling sample plots with no living trees or from the gravel road passing through the area (see Figure 3 and Table 1).

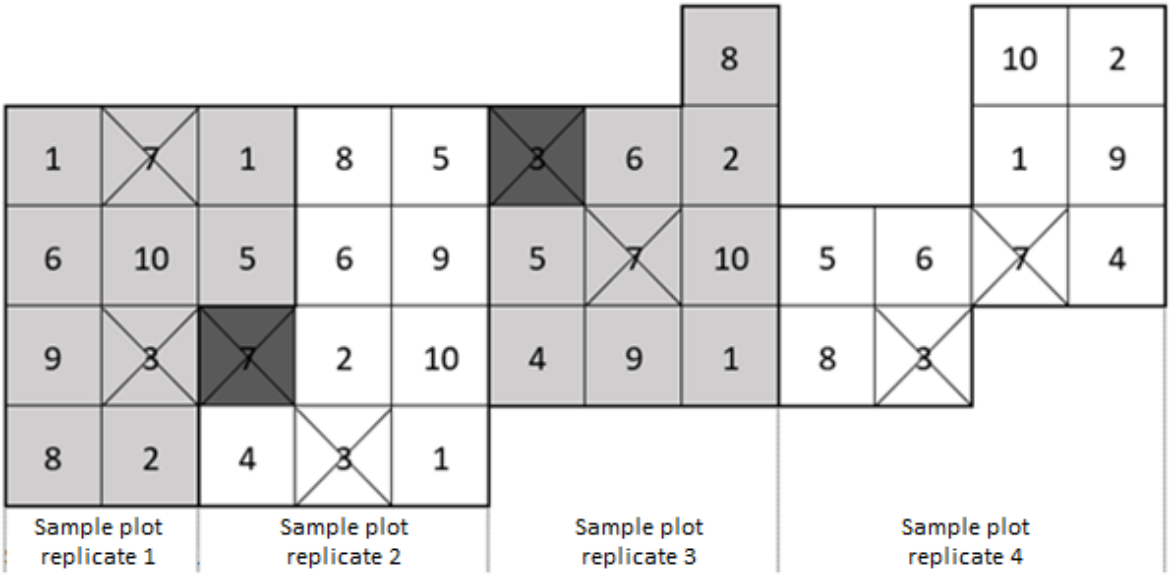


Figure 3. Layout of the intently polluted area on Somerharju restoration site. Numbers in sample plots indicate a planted aspen variety repeated 4 times over the entire area. Cross in the sample plot indicates sample plots, where sampling took place. Dark grey fill in sample plots indicates a spot where control samples were also collected. Light grey fill was used to highlight the four sets of sample plot replicates.

Table 1. Number of samples collected from different sample plot replicates and different sample plots. 4 aspen trees were selected and from their roots two extracts of soil were dug up, one from 0-10 cm depth another 10-50 cm depth and later combined resulting in 4 samples from each sample plots, one for each tree.

Sample plot replicate	Sample plot	N combined samples
Test samples		
1	3	4
	7	4
2	3	4
	7	4
3	3	4
	7	4
4	3	4
	7	4
Control samples		
1	Road	4
2	7	4
3	3	4
4	Road	4

Samples from sample plot replicate 2 were collected on September 10th, 2018 and the rest on October 1st, 2018. On the first sampling time the sampling position in each sample plot was located by moving 5 steps from the north-western corner of the sample plot (upper left in Figure 3) towards the south-western corner and then moving 9 steps towards the opposite border. Within a radius of 2,5 meters of this position 4 samples were collected within 50 cm of 4 different aspen trees. If this method produced a spot with no living trees, the closest spot with living trees

within the sample plot was located. For each of the 4 trees, 1 sample from the topsoil (0-10 cm) and 1 sample from the bottom soil (10-50 cm) were collected to be combined later to a single sample. This was done according to the sampling procedures of Golder Associates Inc. (unpublished) to mitigate the possible bias caused by differences in different pollutant concentrations in different soil depths. Control sample sets 1 and 4 were collected from the road in a straight line along the road with 1 m in between each sample. Control sample sets 2 and 3 were collected from sample plots in the same manner as actual samples this time collecting the samples well removed from any trees.

The samples consisted of soil composed of clay, sand, very fine particles (VFP) or mixtures of the three. VFP was distinguishable by a particularly small soil particle size that enabled it to pass through a 2 mm sieve with little to no help. After the collection, samples were stored in dark at + 4 °C for experimentation the following year after a maximum of 10 months.

Picture 3. Sampling from close to an aspen tree. The sampling spot is within 50 cm of the tree stem to get a better representation of microbial flora strongly affected by the aspen tree. From each hole two samples were taken, one from 0-10 cm depth another from 10-50 cm which were combined as a single sample.

2.2 Sample pre-treatment

Before any measurements, the soil samples needed to be pre-treated and homogenized either by sieving the samples or by drying them.

2.2.1 Sieving and combining samples

All the top and bottom samples were sieved through a clean 2 mm sieve. After sieving, 100 ml of the top and 100 ml of bottom soil samples of each sampling spot were combined into a single sample according to Golder Associates Inc protocol (Golder associates, unpublished) and mixed thoroughly for two hours using a horizontal rotator at a speed of 25 rotations per minute. After two hours, the samples were placed in clean sealable plastic bags and stored in dark at + 4 °C.

2.2.2 Drying

Each combined sample was measured for its inherent water content. This was done for the Microresp™ procedure, water holding capacity (WHC) measurements and soil pH measurements, last two of which required dry samples. A subsample of 6-7 grams of each combined sample was dried in 40 °C oven for 6 days, weighed and the samples for pH measurements were stored into 100 ml glass bottles for later pH measuring. The temperature used in drying caused other substances besides water to evaporate from the samples in at least one case (extra sample not used in the final experiment), where measured amount of evaporated substances exceeded the amount of water possible to be present at WHC 100 %.

2.3 pH measurements

The soil pH measurement was done according to the International Standard ISO10390:2005(E) (International Organization for Standardization, 2005) with the exception that for 10 samples the volume of 6-7 g of soil dried earlier (see 2.2.2) for pH measurement fell below the minimum amount of 5 ml required for the standard. In these cases, the measuring volume varied between 4 - 4,5 ml. 8 of these samples consisted of sand, 1 of a sand-clay mixture and 1 of very fine particles. 5 of these

were control samples, 3 samples close to *P. tremuloides* trees and 2 close to *P. tremula* trees.

2.4 WHC measurements

Water holding capacity (WHC) refers to a soil sample's capability to withhold water in its pores. WHC measurements were done by submerging and draining samples according to SOP M0001 procedure of the University of Eastern Finland, Department of Environmental Science, biochemistry group provided by Tervahauta A. and Liimatainen M. (2018) and the retained water was measured. The measurement was done for 7 samples of different soil types (see 2.5), as measuring WHC of all samples would have been too time consuming. All samples of the same soil type were assumed to have the same WHC as the measured one with a corresponding soil type. Every sample contained one or two of these 7 soil types. In the case of a mixed sample, an average of contained soils' WHCs was used.

2.5 Soil type estimation

The type of soil dominant in each soil sample was estimated by eye and categorized into one of the following five categories according to what soil type or mixture seemed to be most prevalent. The categories were: very fine particle (VFP), sand, clay, sand/clay or VFP/sand with the last two categories being a soil mixture of the soils in the first three categories.

2.6 The Microresp™ protocol

The study site is contaminated with a multitude of HCs and the study of all the contaminants was infeasible, so the degradation of a single HC present in the soil, phenanthrene (Figure 4), was chosen to be studied. PHE is a PAH that consists of three aromatic rings (Royal society of chemistry, 2020). It's oral LD_{50} for rats is 1800 mg/kg and is considered very toxic to aquatic organisms (Sigma-Aldrich, 2020)

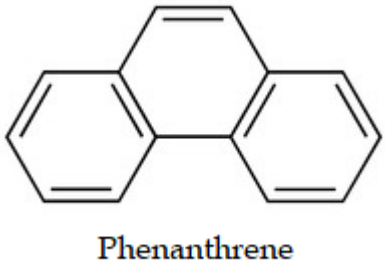


Figure 4. Structural formula of PHE (Royal society of chemistry, 2020)

PHE was chosen for the experiment due to its relatively small molecular size compared to other PAHs. This was hoped to contribute to a measurable substrate degradation with a 6-hour sample incubation which is the normal incubation time with the Microresp™ protocol for easily degradable substrates like glucose. To account for the possibility of an insufficient degradation of PHE within 6 hours, modifications were made to the Microresp™ protocol to allow for a longer incubation. To the best of my knowledge, no research where Microresp™ incubation has been extended to this degree has been reported before.

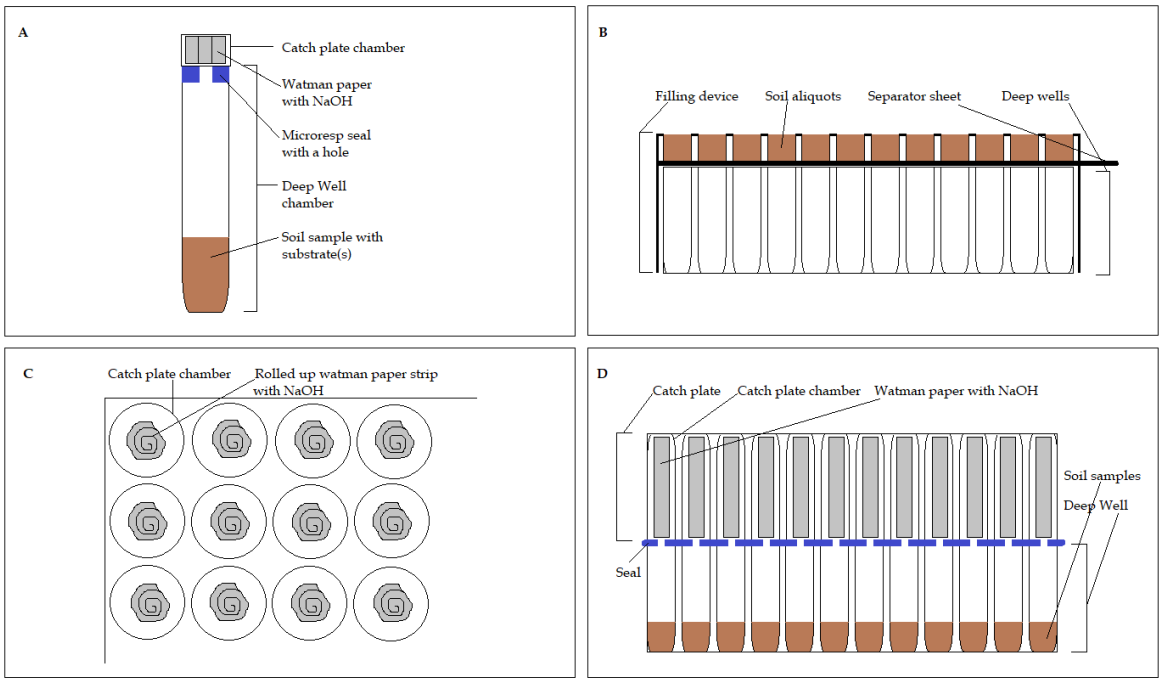
To measure the degradation (i.e. microbial consumption) of PHE the Microresp™ device was used to measure the amount of ^{14}C radiocarbon-labelled CO_2 produced by soil bacteria from ^{14}C-labelled PHE added to the soil samples. The labelled CO_2 is an indicator of the amount of labelled PHE changed into CO_2 through cellular respiration by the microbiota.

The Microresp™ device (Picture 5) consists of a 96 deepwell plate with soil samples (Picture 5 D transparent wells at the bottom) , a seal (blue layer above the wells in Picture 5 D) and a catch plate placed on top of the deep well plate (white plate on top of the device Picture 5 D). The deepwell plate is filled with equal aliquots of soil with the help of a filling device (Picture 5 B). When the plastic sheet between the deepwell plate and filling device is removed, measured aliquots fall to the chambers of the deepwell plate. A seal is then placed on the device covering all 96 wells. On top of the seal another 96 plate is placed. As the Microresp™ measurement

proceeds, microbiota in the soil samples in the lower deepwell plate respire CO_2 which rises to the catch plate through small holes in the seal between the two plates (illustrated in Picture 5 A). The catch plate is designed to either react to the carbon dioxide by changing the colour of an indicator containing agar gel in each well of the catch plate or by neutralizing and thus stabilizing the carbon dioxide to pieces of Watman NO 1 paper containing NaOH (Picture 5 C). In the experimental protocol here, the catch plate with Watman papers with NaOH water solution was used to dissolve, neutralize and stabilize the radiolabelled CO_2 produced by the samples in the deep well chambers according to Formulas 1 and 2.

$$CO_2 + H_2O \rightarrow H_2CO_3 \quad (1)$$

$$2\,NaOH + H_2CO_3 \rightarrow NaCO_3 + 2\,H_2O \quad (2)$$



Picture 5. the Microresp™ device and its working principle. In Picture A an illustration on the principle of CO_2 catch and measure technique, in Picture B the soil filling process, in Picture C the catch plate with Watman NO 1 catch-filter papers, in Picture D a filled device with attached catch plate with papers ready for use. (adapted from Microresp, 2019a; Microresp 2019b)

Both a preliminary test and an extended incubation test were conducted on the samples with Microresp™.

2.6.1 Modifications

The normal Microresp™ protocol needed to be modified from the manual's instructions in several ways. Modifications were made to accommodate for an incubation time of one week instead of the six-hour incubation described in the manual. This was done to ensure a proper degradation of PHE, which was presumed to be more difficult to degrade than the small molecule substrates described in the manual (e.g. glucose).

To achieve a longer incubation time, the concentration and volume of NaOH was increased in the catch plates to allow for more neutralization active NaOH to each well of the catch plate to accommodate for the larger amount of CO_2 produced during a prolonged incubation. The maximum neutralization capacity of the Microresp™ device's catch plate was calculated with Formula 3:

$$Volume\ NaOH_{target\ incubation\ time} = ((hours_{target\ incubation\ time}/6\ h) * 40\mu l)/2 \quad (3)$$

where 40 µl is the Microresp™ manual's designated amount of 2 M NaOH to be used in a 6 h incubation protocol. The amount gained was further divided by 2 as 4 M NaOH was to be used.

To accommodate a larger NaOH volume, the 96-well plates intended to be used as a catch plate were replaced with 96-deep well plates similar to the ones used for incubating the soil samples and these were attached with duct tape. The size of the Watman NO. 1 filter paper in each well of the catch plate was also increased to roughly 14 times larger compared to the manual's instructions.

The volume of the ethanol based PHE substrate solution to be added to the samples was also decreased from the recommended 30 µl to 9 µl to account for the biotoxicity of ethanol.

In the final incubation the designated WHC limit of 60 % of max was purposefully exceeded at one point to ensure a sufficient moisture level in the samples as described in 2.6.3.

2.6.2 Preliminary tests

Several preliminary tests were conducted before the actual experiment to optimise the experimental methods with the Microresp™ device.

Soil samples from the polluted site were incubated with ^{14}C labelled PHE and ^{14}C labelled glucose and degradation was measured after 6 h, 24 h and 3 days of incubation to determine the shortest sufficient incubation time needed for radiolabelled CO_2 production and to see if radiolabelled CO_2 would indeed be produced.

These incubations did not produce adequate results, so the samples without ^{14}C glucose were incubated further with the ^{14}C PHE for a week, after which the degradation was measured again. The samples were further incubated with a new catch plate for another week after this, and the new sample papers were added to the same scintillation bottles containing the first week's papers and the cumulative production of ^{14}C CO_2 was measured. Additional control samples were incubated on a separate plate after the actual samples had been measured.

2.6.3 The final Microresp™ experiment

As the results of the preliminary tests described above proved promising but insufficient, the Microresp™ experiment was repeated.

The 32 test samples and 16 control samples were loaded into three Microresp™ devices with the Microresp™ filling device that delivered less than a gram of each

soil sample to each well designated for it. Each sample had two technical replicates where glucose was added and two technical replicates where a corresponding amount of ultrapure water was added (4 technical replicates for each sample all together).

Water was added to each sample to reach moisture content of 50 % of maximum WHC. It was taken into account that that the addition of reagent solutions later on should not increase the water content above 60 % of maximum WHC. The plates were then incubated according to Microresp™ instructions for 5 days. On the 6th day of incubation 560 µl of 4 M NaOH was added to each paper in the catch plate before adding reagents to the soil samples.

A single glucose solution was prepared to be added to all the samples in an individual volume that would equate 30 mg of glucose per 1 g of soil water in each sample. The amount of glucose to be added to the solution was calculated according to the glucose requirements of the sample with least water and thus requiring least glucose so as not to exceed the desired WHC %or glucose limits (30 mg per g of soil water) of any sample. The volumes of the solution that needed to be added were individualised with an accuracy of 0,02 µl (the accuracy of the pipet) and added to each sample. The differences between the samples' final WHCs due to different amounts of glucose solution added to them was less than 1 % in all cases. The deviations from the target concentration in final glucose content between samples due to pipetting inaccuracy were less than 1 % except with sample 28 (*P. tremula*), where the concentration was 2,12 % below the target concentration.

Then, a target volume of 9 µl of PHE solution (equal to 200 Bq per well) was added to each well. Additionally, 8 empty wells were filled with 9 µl of labelled PHE to monitor the amount of evaporating PHE.

Once the reagents had been added, the catch plate was secured above the deep well plate with duct tape and stored for incubation in room temperature in a plastic box

lined with wet paper towels and a glass petri dish containing ca. 10 g of self-indicating soda lime.

After one week, the filter papers in the catch plate were collected to sample bottles and a new catch plate with fresh NaOH was placed on the Microresp™ device. This was done in case the scintillation numbers of the first week's incubation would prove to be too small. The papers were cut into two with ethanol wiped instruments to diminish the amount of scintillation fluid needed and added to their respective bottles in two pieces. An amount of scintillation fluid sufficient to submerge the papers (ca. 8 ml +/- 3 ml) was added to each scintillator bottle.

In the second week's scintillation, an amount of ultrapure water sufficient to increase the samples' moisture content to WHC 70 % was added. This was 10 % over the maximum defined in the Microresp™ protocol. This was done on the day following the change of the catch plates because while measuring the inherent water in the soil samples, it was noticed that the drying temperatures had caused other substances besides water to evaporate from the samples in at least one case. This might have led to a misestimation of each sample's inherent water content and consequently the final water content during the experiment. Most likely this would have resulted in samples that would be too dry for the bacteria to thrive properly. In fact, one preliminary test had significantly high scintillation count numbers, which could not, at that time, be attributed to anything else but an accidental over addition of water.

To account for the possibility of having a lower than 50 % WHC at the beginning of the first week's incubation, it was decided that water would be added to the samples to reach a 70 % WHC in the second week's incubation.

After the second week's incubation, the papers of the incubation were cut in two and added to the bottles containing the papers from the first week's incubation for a cumulative measurement of 2 week's degradation. Care was taken that the papers would be submerged in the scintillation fluid.

Counts per minute (CPM) were measured from the samples with Tri-Carb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) device (count time 1 minute, assay count cycles 1, repeat sample count 1).

2.7 Gene sequencing

The aim of the genetic sequencing was to produce data on the genetic variance of the bacteria in the soil samples from which a comparable number could be calculated for each treatment (no tree clones, *P. tremula* and *P. tremuloides*).

A measure on the abundance of different kinds of bacteria with different genetic fingerprints was obtained by first extracting the DNA of the soil's micro-organisms using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc, Catalog # 12888-50) according to manufacturer's instructions.

Appropriate DNA sequences, in this case the 16s ribosomal RNA (rRNA), were then sequestered, amplified and sequenced from the samples for their genetic structure. These genes are used as marker genes for living organisms and gathered into clusters according to how similar they are to one another forming what are called operational taxonomic units (OTUs) each cluster being most typical in certain kinds of organisms thus indicating the presence of different bacteria in the samples (He Y. et al, 2015)

The sequencing of 16s rRNA was done with the ion torrent method. Ion torrent is an NGS method in which the ion torrent machine observes the construction of a sample DNA strand by flooding the sample with a single type of nucleotide at a time (ThermoFisher, 2020). If the nucleotide is bond to the DNA strand, a hydrogen ion is released and detected by the machine and the process slowly constructs the sequence of the DNA strand.

The data on the different types of bacteria present was then used to calculate the diversity of each sample as the Simpson's diversity index number and the Shannon

diversity number for a sample by sample comparison. The Simpson's diversity index measures the diversity of a population with regards to the amount of species present and their abundance relative to the abundance of the rest of the species according to Formula 4

$$D = 1 - (\frac{\sum n(n-1)}{N(N-1)} \quad (4)$$

where n is the total number of organisms of a species and N is the total number of organisms of all species (University of Idaho College of Natural Resources, 2020). Shannon diversity also considers the number of individuals of species relative to those on the site but is a little more sensitive than the Simpson's index according to Formula 5,

$$H' = -\sum[\left(\frac{n_i}{N}\right) * \ln\left(\frac{n_i}{N}\right)] \quad (5)$$

where n_i is the number of individuals per a species and N is the site's total number of individuals.

Finally, the different OTU profiles of the samples were also compared for their similarities with regards to their aspen treatment and soil type with a non-metric multidimensional scaling. The details of the sequencing procedure are described in Appendix 1.

2.8 Statistical analysis

The differences in the radiation counts after a two week incubation time were analysed with a generalized linear mixed model (GLMM) with species, pH and glucose set as fixed factors. The linear model was chosen as a statistical analysis method to identify the magnitude of change in PHE degradation each treatment causes. This makes for a more interesting comparison to previous studies. The sample plot number was set as a nested random effect to account for the possibility that the sample plot might have influenced the results of two samplings from within

the same sample plot. The nested version of the model was chosen as the dependence can only occur within a sample plot but does not carry over to the sample plot replicate level. The examination of a random effect was an additional advantage of using a linear model over ANOVA. The GLMM modelling was done using the glmer() function in R in the lme4 package (Bates D. et al., 2015) and the MASS package (Venables and Ripley, 2002). The bobyqa optimizer was used from the optimx package (Nash and Varadhan, 2011; Nash, 2014) for all the models.

The model is presented in Formula 6. The model compares the effects of different treatments to a null sample with no trees and no added glucose.

$$\log(count_{scintillation}) = \alpha + \beta_1 * pH + \beta_2 * glucose + \beta_3 * species_{P.tremula} + \beta_4 * species_{P.tremuloides} + \text{MV } N(0, \Sigma \text{ sampling sample plot}) \quad (6)$$

An AIC comparison was conducted in R to determine if a Poisson distribution or a negative binomial distribution with or without pH included in the model would fit the data better (see Appendix 2) and an ANOVA test from the car library was also performed to see if any differences between the two models was statistically significantly different from one another.

Despite the use of different optimizers in the models the negative binomial models returned a singularity error message. However, out of all the models tested the negative binomial distribution that included pH was deemed to be the best fitting and was more conservative with interpreting the results of all the treatments than the models using a Poisson distribution and had the second lowest of all the AIC values and the difference between the two lowest models was not statistically significant when compared with ANOVA. As pH was deemed a parameter of interest for the experiment and the negative binomial model including it also produced lower singularity compared to the negative binomial model, which excluded pH, the model was used.

3 RESULTS

Results from the Microresp™ protocol were obtained on the third run with longer incubation times. On average under 2 % of total ^{14}C in the samples was transmitted to catch plates. Aspen trees had a statistically significant effect on PHE degradation but did not differ from one another statistically significantly. The controls had a higher bacterial diversity than samples with aspens but had less variation in their diversity profiles between samples.

3.1 Preliminary Microresp™ tests

The shorter incubations of 6 h, 24 h and 3 days proved inadequate to produce results. The extended incubation produced very promising results. The inaccuracies that occurred in this incubation were removed and the final incubation was conducted, and detectable levels of degradation occurred. The radioactive counts gained from the samples represent gross alpha activity from samples and are referred to as Counts Per Minute (CPM). They are a measure of CO_2 produced from labelled PHE.

3.1.1 Radiolabeled substances, short incubation times

The tests with 6 h, 24 h and 3 days incubation times did not produce ^{14}C labelled CO_2, (CPM numbers) that could be discerned from background radiation except in samples to which ^{14}C glucose was added. It was concluded that a longer incubation time for PHE was needed. The samples with ^{14}C PHE were incubated further.

3.1.2 Radiolabeled substances, 1- and 2-week incubation times

When incubated further, the samples from short incubations showed the highest degradation numbers achieved in any test before and after it, well above the background standard. The amount of control samples was, however, insufficient for a reliable statistical analysis. This could not be compensated with an additional plate of control samples incubated later as samples on this new plate that had been

measured before now produced several times smaller numbers compared to the first two runs for unknown reasons and were, therefore, unusable as a control reference. Additionally, there was concern that improper handling of the device whilst filling had caused some soil to sprinkle out of the device resulting in an incorrect amount of reagents and water to be added relative to amount of soil in the device. Having only a single technical replicate for each sample with glucose and one without was also deemed insufficient, so the experimental setting was fixed, and the experiment repeated. The results from this new run are described in 3.2 and used for analysis.

3.2 Final Microresp™ experiment

CPM numbers ranged from a minimum of 76 (control sample, no glucose) to a maximum of 354 which was interestingly produced also by a control sample with no glucose. The mean CPM of all samples was 140,96. All values were at least two times larger than the scintillator device's background standard count (21). The counts from 53 of the 96 samples (technical replicates included) were lower or equal to the average count (127) of evaporation wells containing only labelled PHE. In the case of 9 samples there was a more than 1,5 times difference between the counts of the two technical replicates of the same treatment, the largest of these differences being that of sample 44 (*P. tremuloides*, no glucose), counts 405 and 99 (coefficient 4,09). In the case of 13 samples, the scintillation count measured at two weeks was lower than it was when measured after one week's incubation.

9µl of labelled PHE was administered directly to two pieces of Watman paper and measured, producing an average CPM of 9264 with two replicates. The percentages of labelled ^{14}C transmitted on average from each treatment to catch plate were calculated from this with Formula 7. These results are presented in Table 2.

$$\frac{Average\ CPM_{samples\ in\ a\ treatment}}{Average\ CPM_{9\mu l\ of\ pure\ PHE}} * 100\ \% \qquad (7)$$

Table 2. above: count averages (n=16 for each tree treatment, 24 for glucose and no glucose) gained for each treatment, below: percentage of measured count averages of each treatment compared to the count average of pure PHE in paper.

Pure PHE	**Control**	***Tremula***	***Tremuloides***	**Glucose**	**No glucose**
9264	125	145	154	148	134
Percentages of ^{14}C from the soil samples transmitted to Watman paper					
100,00	1,34	1,56	1,66	1,60	1,45

3.2.1 Effect of aspens

In the GLMM model of scintillation counts, only a small amount of the variation was accounted by the random effect (Table 3). No correlation is distinguishable between the different treatments in Figure 5 either. The outlier zero sample causes a skew to the line of control samples (Figure 5 A). In contrast, the model did show significant difference between the control samples and both the *P. tremula* (*P*=0.027) and *P. tremuloides* (*P*=0,004) aspen species (Table 3). H_0 was rejected and H_1 retained. The model predicted an increase between the control samples and *P. tremuloides* by roughly 44 counts and *P. tremula* by roughly 27 counts (an increase compared to having no trees by factors of 1,4 and 1,25 respectively). This result is however impacted by the unusually high-count value of the outlying control sample. When the analysis is re-run without the excessively high control value the counts change to 48 for *P. tremuloides* (*P*=< 0,001), and 25 counts for *P. tremula* (*P*=0,018). While a

difference between the counts of the two different aspen species treatments was distinguishable (Figure 5 B) it was not significantly different statistically (P=0,3345). H_0 was sustained.

Table 3. Results of GLMM with nested random effects with pH included and negative binomial distribution used as a reference. Log link removed from the estimate and standard deviation. Below the correlation of random effects of collection sample plot and sample plot replicate. Rep:SP stands for repetition:sample plot

Random effects						
Groups	**Name**	**log σ**	**log σ²**	**σ**	**σ²**	
Rep: SP	Intercept	0,12	0,01	1,13	1,28	
Sample plot	Intercept	0,03	0,001	1,04	1,07	
Fixed effects						
Treatment	**Log estimate**	**Estimate**	**log σ**	**σ**	**z value**	***P***
Intercept	4,7	109,6	0,08	1,09	56,8	<0,001
Centered pH	0,19	1,2	0,19	1,21	0,99	0,321
Glucose	0,09	1,1	0,05	1,05	1,79	0,073
P. tremuloides	0,34	1,4	0,12	1,13	2,86	0,004
P. tremula	0,22	1,25	0,1	1,1	2,22	0,027

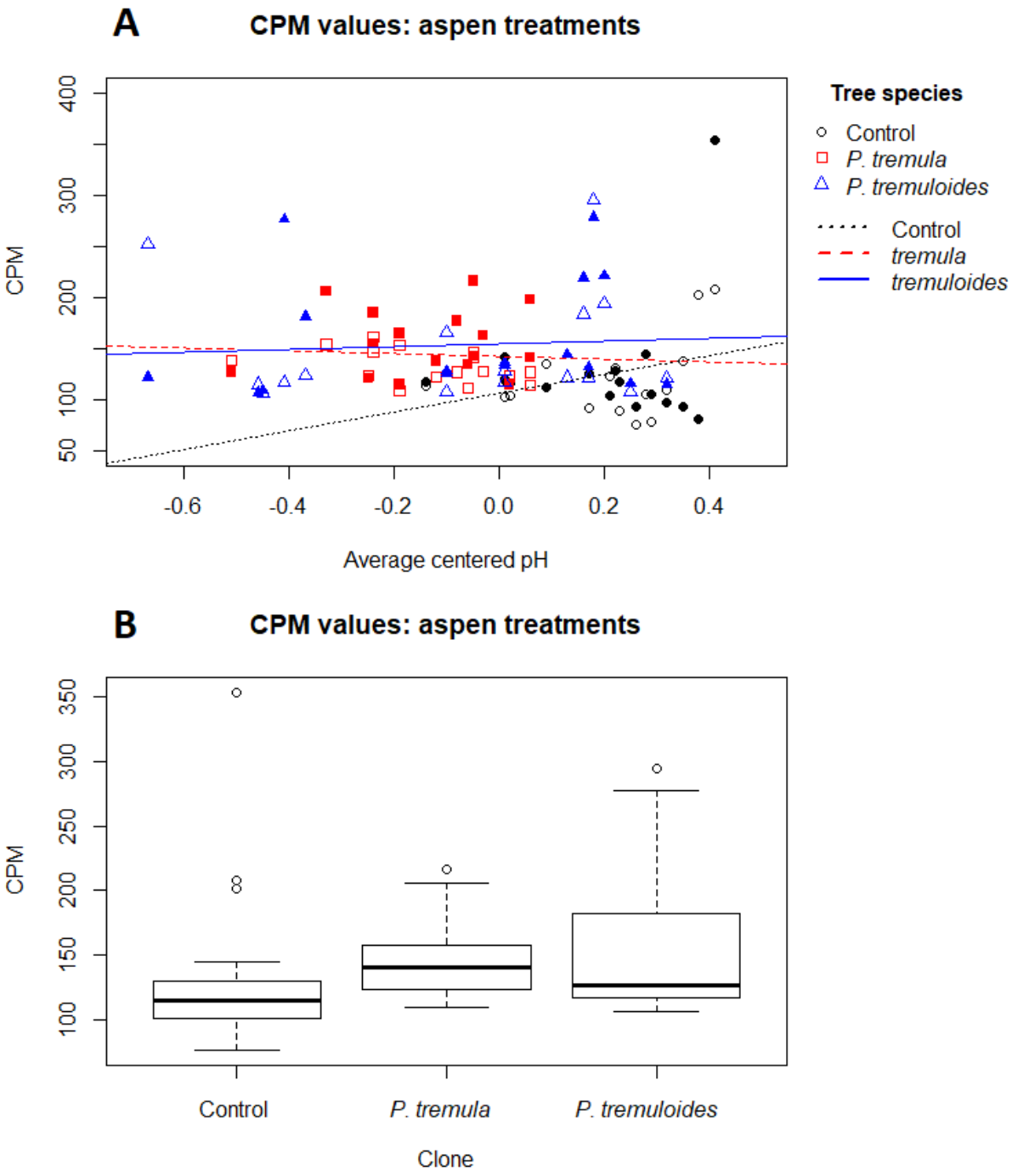


Figure 5 A. the scatterplot of scintillation counts with each tree species and control samples relative to calculated average pH of samples. Filled symbols indicate samples containing glucose. B. the box plot of the effect of different aspen species on the scintillation count. The upper and lower boundaries of the box represent Q3 and the Q1 of observations respectively, the thick line in the middle is the median. The upper whisker's position is either the largest observation or Q3 + 1,5 * range between Q3 and Q1 depending which is smaller. The lower whisker is equal to Q1 - 1,5 * interquartile difference or the smallest observation depending which is smaller.

3.2.2 pH and glucose

The GLMM analysis concluded that neither pH nor glucose had a statistically significant effect on CPM (P=0,3208 and P=0,0731 respectively) as can be seen in Table 3 and Figures 5 A and 6. H_0 was sustained in both cases. pH increases scintillation counts by roughly 23 counts and glucose by 10 counts. If the outlier control sample is removed the corresponding numbers are 8 counts for glucose (P=0,083) and less than 1 count for pH (P=0,969)

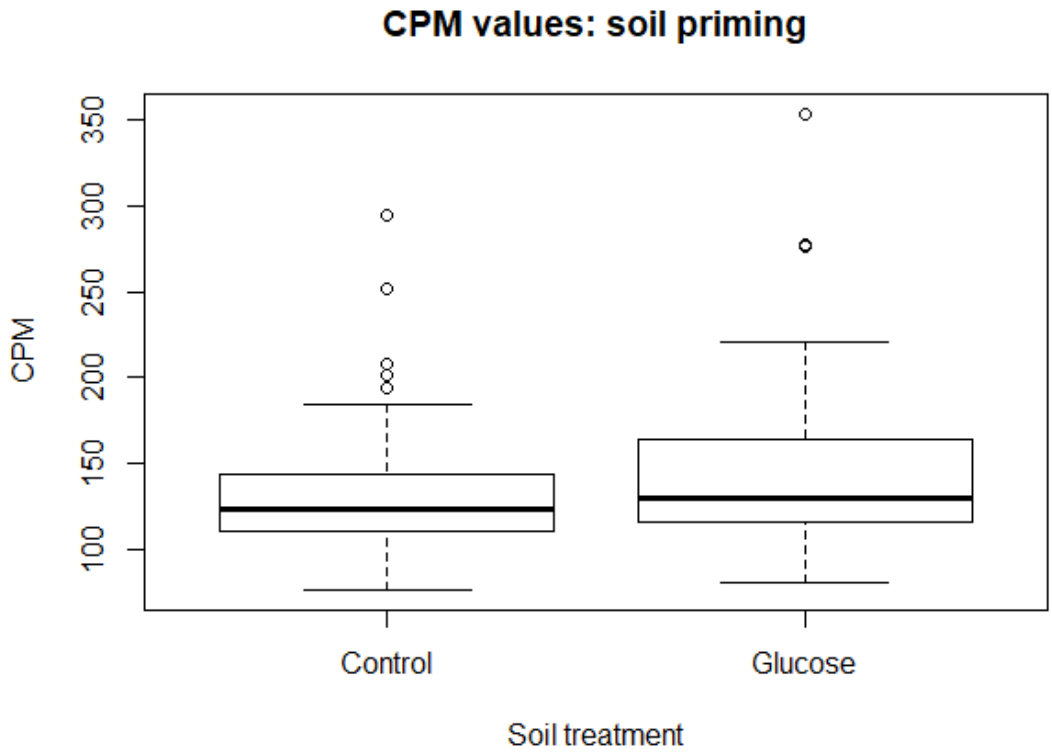


Figure 6. The box and whisker plot of glucose containing samples and those without glucose. Boundaries of whisker boxes by the same principles as in Figure 5.

3.2.3 Alternative GLMM models

The results of GLMM models with a Poisson distribution were also consulted to account for the discrepancies in the negative binomial model that was used (Appendix 2, Tables 5 and 6). The Poisson model that included pH along with the rest of the variables and was the most fitting according to AIC from the Poisson

models had no singularity issue like the negative binomial model but was poorer fitting according to AIC. In this model all the treatments had a significant impact on degradation both when the outlier observation was included and when it was excluded (see Appendix 2). The results of the negative binomial model which included pH as a variable were used to interpret the differences instead as these results were clearly more conservative and the model had a significantly better fit with AIC. Only the aspen treatments produced statistically significant results with this model both when the outlier was included and when it was excluded. The best fitting model according to AIC, the negative binomial model without pH, dubbed the results of *P. tremula* as borderline significant both when the outlier was included (P= 0,052) and when it was excluded (P= 0,057). As pH was of interest to the experiment and the AIC difference between the used model and the best fitting was 0,87 the model containing all the data was used.

3.3 Gene sequencing and diversity

The OTUs of soil samples were analysed for local diversities, and similarities of OTU profiles (which OTUs, i.e. which bacteria, were present in soil samples) relative to aspen species treatment and soil type of the sample. One sample with *P. tremula* treatment and one with *P. tremuloides* treatment had too low OUT counts during the subsampling necessary for the diversity analysis used in this research so they were dropped out but the difference in numbers between treatments was deemed to be of no consequence to the interpretation of the results. The samples omitted both had sandy soil.

The results are presented in Figures 7, 8 and 9. The control samples express a slightly higher diversity with both measurements for local diversity (Figure 7). Also, diversity within treatment seems to be rather more variable in samples with trees particularly in the Shannon diversity.

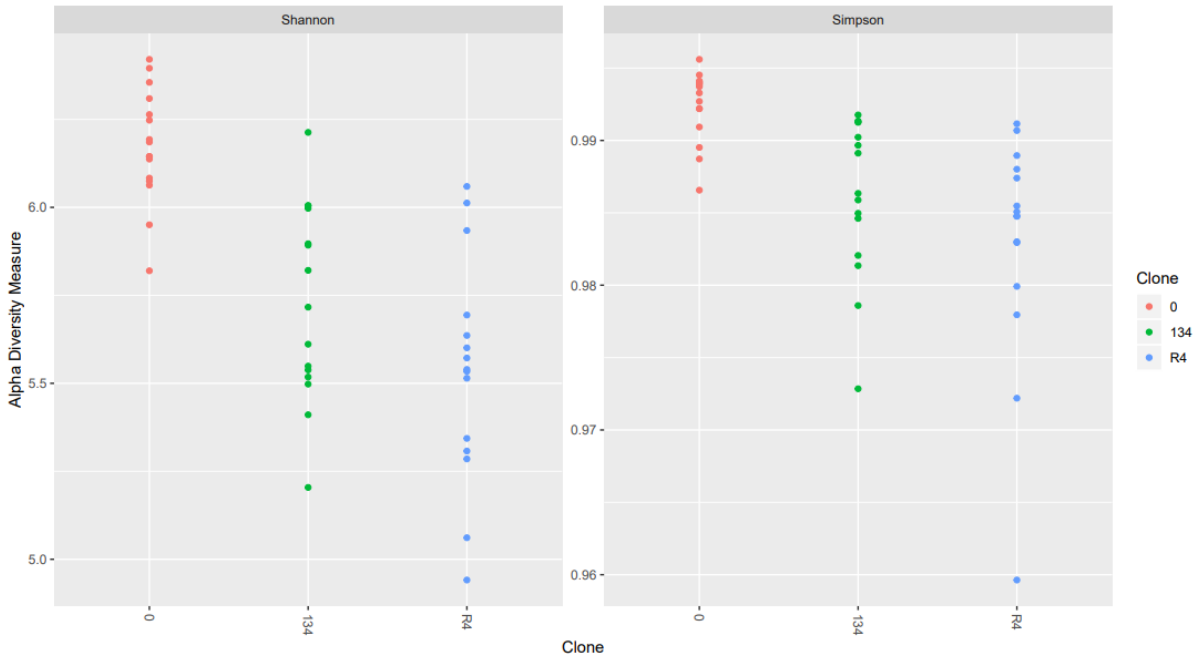


Figure 7. Shannon and Simpson diversities presented on the y-axis according to bacterial OTUs of soil samples presented on the x-axis according to aspen species. Aspen treatments respectively from left to right in both Figures: control, *P. tremuloides* and *P. tremula*. n=16 controls and 15 for *P. tremula* and *P. tremuloides* treatments.

From the non-metric multidimensional scaling (NMDS) plot we can see that some genetic similarity indicating grouping within samples is detectable (Figure 8). It seemed that despite their higher diversity the control samples seemed to be most tightly clustered than the samples near trees, suggesting higher between sample similarity. In contrast the samples near trees were more spread out and for the most part overlapping, suggesting overall differences between individual trees was higher than between species. Interestingly the samples with VFP soils seem to form a loose cluster on the left side of the plot, indicating soil type had a greater influence on these samples' bacterial composition than aspen species did.

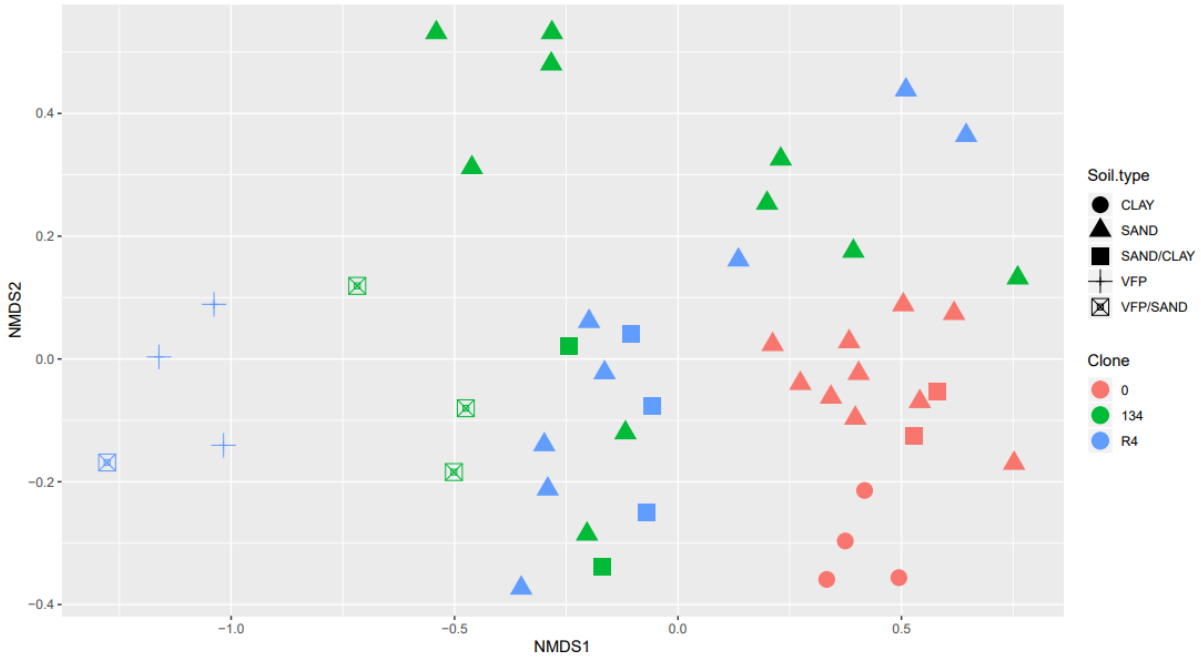


Figure 8. Similarity of samples according to OTUs identified. Every sample is represented by an individual dot. Closer proximity indicates a more similar bacterial profile between samples. Numbers 0, R4 and 134 represent control samples, samples near *P. tremula* and samples near *P. tremuloides* treated samples respectively. n=16 controls and 15 for *P. tremula* and *P. tremuloides* treatments.

The bacteria profiles do not indicate huge differences in the main phylum in the three groups (Figure 9). However, one is able to discern that the profiles of aspen treated groups are more similar to one another than they are to the control group. *Proteobacteria* seem to be the most dominant bacteria phylum in all the groups and *Bacteroidetes* phylum is a little more common in aspen treated soils. H_5 receives some support.

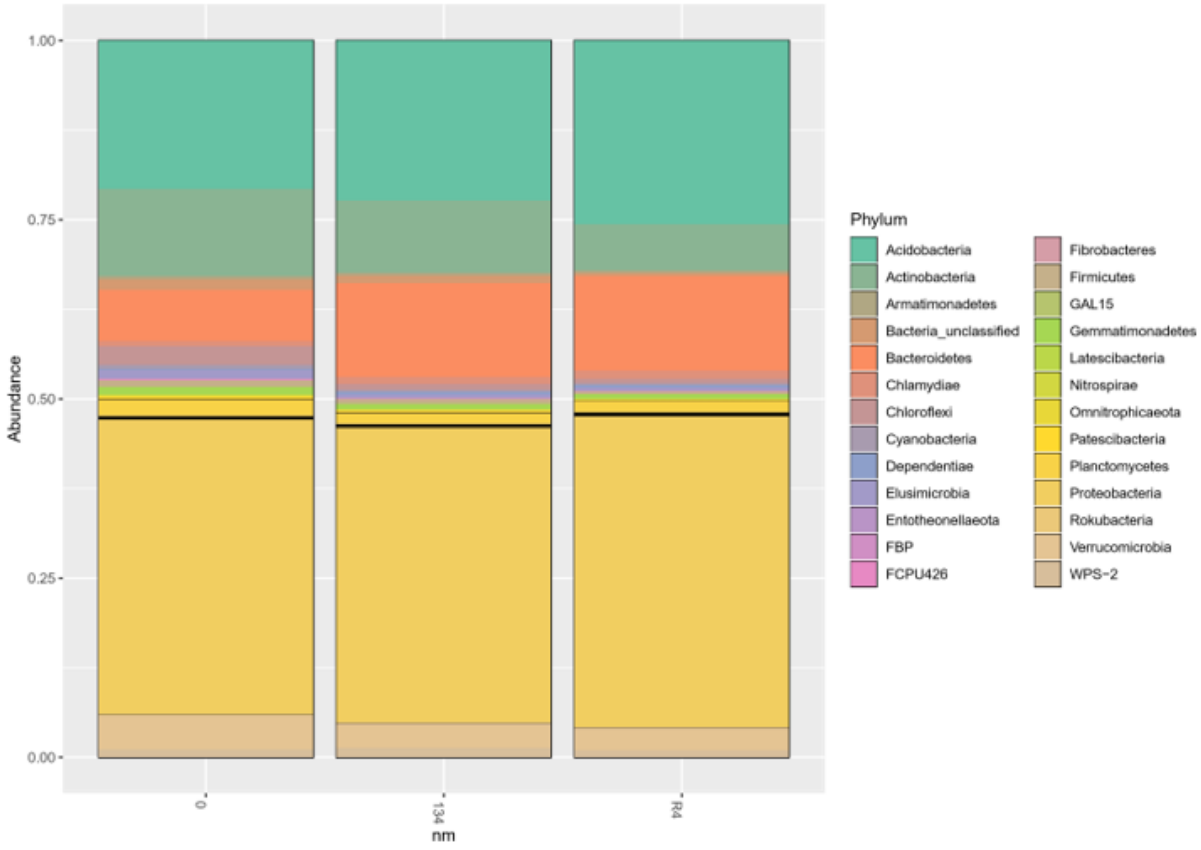


Figure 9. Relative abundance of different bacteria types within samples with different aspen trees (columns from left to right: control, *P. tremula* and *P. tremuloides*).

4 DISCUSSION

The control samples showed higher local diversity of the bacterial community and this trend was more consistent between samples than the diversity in samples collected near trees. The incubation setting produced scintillation numbers for PHE degradation in a two-week incubation which was a successful extension of the incubation time described in the Microresp™ manual.

I found statistically significant differences in the amount of respired radiolabelled CO_2 in soil samples close to aspen trees but found no a statistically significant difference between the amount of respired PHE between the different aspen species.

4.1 The effect of soil pH on CPM

The slight increasing trend in PHE degradation with the increase of pH was not statistically significant. In a previous study with a single strain of bacterium *Pseudomonas putida* under laboratory conditions Shin et al. (2004) found that pH did not have a "dramatic effect" on the cell growth of bacteria using PHE as their nutrition but did notice that the degradation was at its average highest at pH 6. In contrast, Kästner et al. (1998) reports a tenfold increase in the amount of anthracene and pyrene degradation with water and inoculated bacteria when the pH of their soil samples was increased from initial 5,2 to 7. Dibble and Bartha (1979) also reported an increase in oil-based pollutant degradation with the increase of pH. In this thesis no significant increase in degradation with the pH range between pH 4,9 and 5,98 in samples was detected. The range of pH variation was, however, not as high in this research as it was in the research of Kästner M. et al. and no sample had pH as high as 7. The possibility of pH having an impact on PHE degradation should not be wholly excluded and could be tested in the future by testing soils with wider pH ranges.

4.2 The effect of added glucose on CPM

The addition of glucose did not have a statistically significant increasing effect on PHE degradation in the samples. When evaluating if my finding is supported by previous research, one needs to take into consideration the fact that the samples used in this experiment were not pure cultures of any microbial strain but rather a natural mixture of different soil microbes. Several authors have described the responses of different pure soil microbial strains to the addition of glucose relative to the microbes' PHE degradation affinity. For example, Terrazas-Siles et al. (2005) showed that the white rot fungus strain of *Bjerkandera sp.* could generate PHE degrading enzymes better in lower concentrations of glucose than in higher concentrations. Another study by Ye et al. (2018) found that the addition of glucose to soil samples collected from a petroleum contaminated Shengli Oil field increased the degradation of PHE by as much as 69,29 %. It would have been interesting to see what kinds of results different glucose concentrations would have produced in the experimental setting of Terrazas-Siles et al.'s (2005). If a high glucose concentration resulted to lower PHE degradation in their earlier experiment, what was the reason? Hypothetically, this could be because the fungi preferred to use glucose over PHE as their primary carbon source in their experiment at high concentrations. If so, perhaps a lower concentration would have had a different effect, particularly once glucose had been depleted from the soil. If this was the case, one could reflect on it and postulate that different glucose concentrations might have had different kinds of impacts on PHE degradation in my study as well. Other concentrations of glucose would have shed light to this. Failing this, it can at least be said that the addition of glucose in a concentration that equates to 30 mg/g of soil water in the samples in this experiment did not clearly decrease PHE degradation as it did in Terrazas-Siles et al's (2005) research. My results concur with other research where no trend between glucose addition and pollutant degradation was found, such as that of Qiu et al. (2007) who found no increase in the degradation of p-nitrophenol with the addition of glucose. In future studies with the experimental setting presented here it would be interesting to perhaps try either a

higher initial concentration of added glucose or add several doses of glucose to the samples throughout the experiment.

4.3 The effect of planted aspens on CPM

The findings of this study showing increasing effects of *P. tremula* on the degradation of PHE are particularly exciting as no such results have, to my knowledge, been previously reported in this species and with this type of pollutant. Table 4 summarizes a few other research settings and their degradation success with different plants. These studies are done without the Microresp™ device as according to my knowledge no studies similar to mine in the field of phytoremediation have been conducted with the device.

Table 4. results of other experiments on PAH removal with different plants compared to this study. First six rows describe the difference between plant treatment and no treatment, rest of the rows present numbers gained from both treatment and control in terms of pollutant degradation.

Publication	Pollutant	Plants used	Duration	Degradation
This thesis	PHE	*P. tremula*	14 days	25 % more than control
		P. tremula x tremuloides		40 % more than control
Cheema et al. (2009)	PHE	*Festuca arundinacea*	65 days	1,88-3,19 % more than in control

	Pyrene			8,85-20,69 % more than in control
Liu et al. (2014)	PHE	*Vallisneria spiralis*	54 days	15,2-21,5 % more than in control
	Pyrene			9,1-12,7 % more than in control
Mueller and Shann (2006)	Several different PAHs	Several North American trees including hybrid poplar	over the course of 365 days	No effect
Lee et al. (2008)	PHE	Native Korean plants	80 days	>99 % removed, 99 % with control
	Pyrene			77-94% removed, 69 % with control

Xu (2006)	PHE	*Lolium perenne L. Zea mays L.*	60 days	ca. 99 % removed, ca. 77 % with control
Sillanpää (toim. 2007)	Oil based pollutants	*P. tremula* × *tremuloides l. P.* × *Wettsteinii*	5 years	No effect

The percent differences between treatments and control yielded in my experiment are larger than in any of the studies listed above, and this study is the first that describes an increase in degradation compared to control whilst using plants of the poplar family. There may be several reasons for this. In the study of Sillanpää (2007), which yielded no results with *P. tremuloides,* the experimental setting was conducted *in situ* instead of in a closed system like in my study subjecting the results to variables that could not be standardized, such as pollutant movement in the soil due to natural phenomena, such as water flow. The results of our studies may thus not be wholly comparable. On the other hand, the study of Mueller's team, which similarly produced no results with trees from the poplar family was conducted in plant pots, effectively creating a closed system more like my own. As Mueller's study reported no degradation at all, the results we gained are highly contrary considering not only the positive results but also the size of the effect.

The 25 % degradation I observed for *P. tremula* is somewhat similar to some other results, such as those of Liu H. et al. (2014) who reported 15,2-21,5 % greater degradation of PHE compared to control while using *Vallisneria spiralis,* which is not a tree like *P. tremula.* Euliss et al. (2008) gained much higher degradation percentages in petroleum hydrocarbon restoration with grassy plants than they did with willows (*Salix exigua*) or poplars (*Populus spp*.) indicating another clear

difference suggesting that research using shrubs or grasses and research using trees might produce different results.

However, despite these differences to previous research, my findings clearly indicate that aspens do cause an increase in PHE degradation, warranting a confirmation of the study, perhaps under conditions more similar to those used by Mueller et al. (2006). Longer incubation times in other studies might also have contributed to a situation where artificially spiked soils were depleted of most of their pollutants before measurements were made resulting in less accentuated differences between treatments and controls as may be the case in Lee et al.'s (2008) study.

To the best of my knowledge this study was also the first to describe the differences in PHE degradation between *P. tremula* and *P. tremuloides*. The lack of statistically significant difference between the degradation increasing effects these two species was interesting. The use of endemic *P. tremula* aspens could, for one, offer an alternative to the use of *P. tremuloides,* if concern were to arise that hybrid aspen might act as a harmful alien species in our environment, especially since they have no statistically significant difference in their degradation increasing capabilities. Another merit of using the endemic *P. tremula* over *tremuloides* is its considerable benefit to Finnish ecological diversity. Studies have shown that *P. tremula* hosts almost as many red-listed species as *Picea abies* and *Pinus sylvestris* in the Finnish forests (Tikkanen et al., 2006). The same study revealed that *P. tremula* hosts the greatest proportion of critically endangered species among the tree species researched. When we also consider the concern expressed by Kouki, Arnold and Martikainen (2004) over the decline in *P. tremula* numbers in Finnish forests we see the potential value of using *P. tremula* in phytoremediation as a means to increase the abundance of an important host species.

This study differs from previous work by the shorter timescale of the experiment. Unforeseen factors might have had an impact on the degradation over time so any

conclusion drawn from the results here should be regarded with a certain amount of caution when hypothesizing effects of aspens on PHE degradation in a long-term experiment. A short study time might give a more optimistic picture of aspens' degradation enhancing capabilities. On the other hand, the absence of trees and their rhizosphere effect on the soil samples during the 2-week measurement might contribute to smaller numbers than would be gained if trees were present.

The soil samples used in this experiment also inherently contain unverified and plausibly unequal amounts of HC pollutants which in turn might have different level effects on different samples. HCs such as petroleum have toxic effects on micro-organisms (Tang et al., 2011) and a higher concentration of e.g. alkanes of the HC family in the soil correlates with the concentration of alkane degrading genes in the microbiota (Powell et al., 2006). There is hence reason to believe that the microbiota in different samples may be different with regards to their bacterial concentration and degradation capabilities due to different pollutant levels. The differences in bacteria's PHE degradation efficiency between different tree species could thus be accounted for a difference in the abundance in hydrocarbon degradation genes produced in response to different environmental conditions between different sites. However, as the differences in samples did have a systematic and logical nature with treated samples having higher scintillation counts, this seems less likely.

The condition and rate of growth of the aspen trees varied also considerably from one sampling sample plot and indeed from one sample spot to another (tree sizes between 50 cm and 3 m). The condition of the aspen can most probably be thought to reflect on the condition of the rhizoflora and in turn to have an impact on the degradation observed from the sample collected from its rhizosphere. If this is the case, at least some part of the results in this study may be attributed to the differences in the condition of the trees the soils of which were sampled, a variable that could not be standardized.

The best fitting statistical model, which excluded pH from the variables indicated only the effects of *P. tremuloides* statistically significant. As pH was of interest to the results of this study, the model including it, the second best according to AIC, was used to interpret the results. As this second-best model indicated *P. tremula* to have significant effect and even the best fitting model suggested it to be borderline significant, it has most likely had an impact on PHE degradation. A certain amount of caution should be exercised though whilst interpreting this and verifying studies are required to properly assess *P. tremula's* potential in PHE degradation.

4.4 Genetic diversity

The most common bacterial phylum detected in the samples was *Proteobacteria,* which was also the case in the research of Mukherjee et al. (2013) on rhizosphere bacteria in oil polluted soils. With regards to the bacterial diversity, the results presented here are somewhat conflicted. In the control samples the within sample diversity of bacterial phyla was generally larger than in samples sampled near trees. On the other hand, the multivariate analysis revealed diversity was much more clustered in all the control samples, meaning they seem to have similar bacterial profiles, whereas the rhizosphere samples varied more between samples in their bacterial community structure. As some of the control samples were collected from the gravel road running between the experimental area this trend might be according to the findings of Pui-Yi et al. (2001) who found that pollution lowered diversity. If the gravel on the road is less contaminated (perhaps it has been imported from outside the remediation area) this could be the case. The control samples were generally clustered according to the bacterial diversity, however, so if it is true that some spots were less polluted than others, it is surprising to see similar kinds of bacterial profiles in all controls. As measurements for the inherent pollutant concentrations were not done, however, the results regarding this matter remain inconclusive.

Samples collected near trees had more dissimilarities in their bacterial profiles between individual samples. A greater bacterial diversity with trees compared to control samples like what was observed in the research of Mukherjee et al. (2013) did not occur. Rather, a less diverse colony of bacteria in the Shannon diversity index was found in treated samples compared to controls. The divergence between my study and Mukherjee et al.'s might be due to a difference in rhizosphere soil collection; in my study the soil was dug from near aspens whereas in Mukherjee et al.'s study the soil was collected by extracting the plant and collecting the soil in direct contact with its roots. On the other hand, Mukherjee's research group found a clear activation of *Betaproteobacteria* with the addition of oil. Combining this to the finding in my research of lesser local diversity contributing to higher degradation one could postulate that diversity is of lesser importance than the activation and contribution of a single microbe phylum in PHE degradation.

The hypothesis of the importance of a single microbial phylum over high diversity gains support from two other studies. Shahsavari et al. (2015) found that in a study of phytoremediation with wheat, both control soils and planted soils contained bacteria with a the *nidA* gene related to PAH metabolism, but in plant treated samples the quantity of gene copies was several times larger than in control samples. Li et al. (2019) had similar kinds of results in their study; PHE degradation correlated with functional gene abundance but it did not correlate with the total 16S rRNA genes i.e. total genetic diversity. According to these studies, abundance of specific bacteria is more important than general diversity. The study of Tam and Wong (2008) has some interesting parallels. In their study of sediment PAH remediation, inoculated bacteria did not survive the contest with indigenous bacteria in the sediments, and yet, the sediments were able to support remediation with the indigenous bacteria. This would further support the notion that microbiota diversity is not of paramount importance to remediation but supporting the correct types of bacteria is. Further study with inoculations of a single bacterial strain might shed light to this.

The soil type of the samples could also have had a more prominent effect on OTU similarity between samples. The soil type of a sample has been reported to have an effect on bacterial colonization, gene abundance and gene expression in the rhizosphere during a phytoremediation process and high values in these factors been observed at least in loamy soils (Afzal et al., 2011). The different soil types thus might have had a considerable impact which could not be eliminated on individual samples. However, due to the relatively small number of samples and the diversity of soils at the site (often occurring across a gradient) it is hard to make predictions about the impacts of soil type on degradation based on the results of this study. Additionally, only the VFP samples appeared to cluster according to their sequencing data.

There may have been other technical issues including cross contamination during handling, particularly sieving, that may have biased the results. The long storage time may also have had an impact on the diversity of the samples; despite the cold storage some succession in the microbiota probably occurred naturally. A certain amount of care needs to be taken whilst interpreting the results on the composition of the microbiota. The PowerSoil gene sequencing kit used for all samples was also outdated (01/2016).

4.5 The modified Microresp™ protocol

The testing done on the Microresp™ procedure gives promising possibilities as an alternative method to other ways of measuring soil pollutant degradation. The incubation time of the Microresp™ method has previously been prolonged e.g. by Bonner M. et al, (2018) whose experiment contained an incubation of soil samples with several different substances such as glucose, phenols and amino acids for 12 hours whilst using the Microresp™ colorimetric method. The results presented in this thesis work suggest that an even longer incubation time produces results as well with the Microresp™ test for radiolabelled substances.

Tests for the degradation of PHE as the substrate have been conducted in the past and experimental settings have yielded results with shorter incubation times than the two-week incubation presented here as well. For example, in the research of Bengtsson et al (2010) adequate results for the degradation of ^{13}C radiolabelled PHE were gained in a 24 hours incubation repeated 7 times and measured with the aid of mass spectrometry and gas chromatography. The modified Microresp™ method presented in this thesis, however, offers an optional way for measuring degradation of radiolabelled substances in the soil without the need for the specialized equipment if it is unattainable or otherwise not applicable.

The prolonged incubation in this experiment may potentially have resulted in anaerobic conditions in the wells. Although some bacteria have been reported to degrade PHE under anaerobic conditions (Chang, Shiung and Yuan, 2002) and the experiment produced results, such conditions in the experimental setting may not represent those of the natural environment in the best way. Also, the study of Liu et al. (2014) concludes that the main mechanism for phytodegradation is through oxygen release by tree roots, which supports the high oxygen demand of bacteria needed for PAH degradation. A similar kind of trend was discovered by Li et al. (2019) who found that root exudates alone did not increase PHE biodegradation, but the presence of a rhizosphere did. A proper aeration of the device combined to a more regular change of fresh catch plates might help to overcome this. Incidentally, as insufficient moisture was ruled out as a possible reason for the high CPM numbers in the first 2-week incubation, another possible reason for them might have been the more frequent opening (and aeration) of the device.

Microbiota are also known to bind the ^{14}C labelled carbon of the PAH naphthalene in their biomass instead of respiring it (Rockne et al. 2000). Although some labelled PHE was clearly respired in this experiment, the experiment might not accurately present the total amount of PHE degraded because of possible binding to biomass. Additionally, there is a possibility that different bacteria have different profiles in how much ^{14}C they bind to their biomass and how much of it they respire as labelled

CO_2. Boshker et al (1998) have described how different microbes have different profiles in the amount of ^{13}C they bind to their biomass. In their study, sulphate-reducing bacteria were the primary binders of ^{13}C acetate in estuarine sediments. Thus, it would hypothetically have been possible to discover different degradation profiles than those reported here for different treatments – particularly in the case of aspen treatments as differences in their bacterial profiles were observed –if measurements had been done for labelled PHE still extant in the sample at the end of this experiment rather than respired ^{14}C.

During the Microresp™ experiment, several wells were also designated to the measuring of PHE that might volatize spontaneously. These wells, however, produced considerably high CPM numbers, at times exceeding those from wells containing both PHE and samples. This could possibly be accounted by the sorption of PHE to soil particles in the wells that contained both PHE and soil (Ping et al. 2006) which most likely prevented it from evaporating and thus causing bias in estimating how much PHE is evaporated under normal conditions. To prevent the bias in estimating evaporated PHE, autoclaved soil samples could have been used for the evaporation control wells to give a clear idea of the amount of non-biologically volatilized PHE as described by Renault, Ben-Sassi and Bérard (2013). The long incubation time could, however, have enabled new bacterial growth so certainty of unbiased results could not be guaranteed even with autoclaved soil. One possibility could be to arrange a separate deep well plate for all the PHE evaporation controls and autoclave all the equipment along with the soil where PHE for evaporation control is then added. This control Microresp™ should not be opened before the end of the experiment to minimize the amount of microbes infiltrating the autoclaved control soil. If total amount of PHE removed from the soil needs to be determined methods such as those described by Powell et al. (2006) should be employed to determine the amount of labelled material bound to soil biomass.

CONCLUSION

Modifications of the Microresp™ device protocol enable results in prolonged incubations with substances that are considerably more difficult to degrade than those described in the device's manual. If sufficiently reliable results can be produced with a modified Microresp™ protocol it will be a valuable option as an alternative to other phytoremediation study methods that require even months study time. The short term degradation study I have conducted presents exciting new information on the more immediate effects of the rhizosphere on pollutant degradation in a field where degradation has usually been measured only after months of plant growing.

The possible effects of glucose or soil pH on PHE degradation gained no support in this study but previous research suggests further study on these factors might help improve the phytoremediative process described here further.

This study produced exciting new information on the phytoremediative capabilities of *P. tremula* and *P. tremuloides* on PHE. Contrary to previous findings, these trees may have significant phytoremediative capabilities which do not differ significantly from one another, supporting the use of *P. tremula*, and thus increasing the amount of a declining key species in Finnish environment. Further research is, however, required to verify this.

The study here has also shed light to the function of microbiota during phytoremediation. If biodiversity is not of great significance to remediation future studies could be directed into finding ways to improve the activity of bacteria crucial to each remediation process.

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APPENDIX 1 USED NGS METHODS BY HOPKINS D.

The NGS methods following the extraction of DNA from the soil samples used for researching the metagenome of the bacteria in the researched soil samples. The following is a quote from dr. David Hopkins who executed the procedure:

"After this…" (extraction) "…extracted DNA was used for 16s ribosomal RNA (rRNA) amplicon sequencing, which targeted the variable region 1 and 2 of the 16S rRNA gene (Hopkins D., 2020). The methods used consisted of two PCR reactions. In the first PCR, the region between 27F (AGAGTTTGATCMTGGCTCAG) and 338R (TGCTGCCTCCCGTAGGAGT) primers were amplified by 10 minutes at 95°C of initial denaturation, followed by 25X cycles of 30 seconds at 95°C, 30 seconds at 45°C and for 30 seconds at 72°C, which was followed by a final 10 minutes elongation phase at 72°C. The second PCR reaction, these primer products then had their barcodes (unique to each sample) and the M13 and P1 adaptors attached using the same PCR conditions as before, but with only 10X cycles. All PCRs were run on a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories) using syber green polymerase master mix (Thermofisher). Sequencing was done with an Ion Torrent (Life Scientific) Next Generation Sequencer (NGS), using an ion314 chip.

The sequence data was then processed in operational taxonomic units (OTUs) using mothur (https://www.mothur.org/, 2019) and the Svila 132 database. The analysis of OTU diversity was done using Simpson and Shannon diversity indexes and explored using non-metric multidimensional scaling (NMDS) using the phyloseq function (McMurdie and Holmes, 2013) in R (Rstudio Team 2016) and the plots where also created in R."

APPENDIX 2 ALTERNATIVE STATISTICS INTERPRETATIONS

Table 5: The summary of GLM using a Poisson distribution with pH and outlier included.

	Estimate	**s**	**z value**	**Pr(>|z|)**	
Intercept	4.65	0.09	49.80	$<2{,}0*10^{-16}$	***
Average centered pH	0.25	0.07	3.56	0.0004	***
Glucose	0.10	0.02	5.71	$1{,}15*10^{-8}$	***
P. tremuloides	0.56	0.04	13.52	$<2{,}0*10^{-16}$	***
P. tremula	0.14	0.05	2.88	0.004	**

Table 6: AIC values of the four different models tested for the data when the outlier is included.

Model	df	AIC	deltaAIC	weights
Negative binomial, pH excluded	7	981.33	0.00	0.61
Negative binomial, pH included	8	982.20	0.88	0.39
Poisson with pH	7	1554.55	573.22	0.00
Poisson without pH	6	1565.26	583.93	0.00