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Occurrence of *Phytophthora* species in soils, and functional analysis of soil microbiomes on Biolog EcoPlates

- A study on commercial soils, and forest and urban soils

Catharina Schmidt

Master thesis • 30 credits
EUROFORESTER
Master Thesis no. 327
Alnarp 2020

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Catharina Schmidt

Supervisor: Johanna Witzell, SLU, Southern Swedish Forest Research Centre
Examiner: Henrik Böhlenius, SLU, Southern Swedish Forest Research Centre

Credits: 30 credits
Level: Advanced level A2E
Course title: Master thesis in Forest Science
Course code: EX0928
Programme/education: Euroforester Master Program SM001
Course coordinating department: Southern Swedish Forest Research Centre

Place of publication: Alnarp
Year of publication: 2020
Cover picture: Johanna Witzell
Online publication: https://stud.epsilon.slu.se

Keywords: *Phytophthora*, *Fagus sylvatica, Quercus robur,* soils, Biolog EcoPlates, metabolic diversity

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Abstract

Tree pathogenic *Phytophthora* species have been identified as a threat to health and stability of forests und urban trees. In Sweden most *Phytophthora* species are non-native and potentially invasive, and thus it is important to control their spread. To identify the presence of *Phytophthora* species in soil from symptomatic forest stands, urban trees and commercial soils, laboratory analyses using a polymerase chain reaction (PCR) with the internal transcript spacers (ITS) pair ITS1oo/ITS4 were conducted. The results revealed that *Phytophthora cambivora* was present across all habitats and in commercial soils. Furthermore, *P. plurivora* was detected in one soil sample from an urban area. Indications of other *Phytophthora* species were found as well but could not be confirmed on species level. The results suggest that *Phytophthora* are widely present in southern Sweden and provide new evidence that commercial soil can be an important pathway spreading these pathogens. Biolog EcoPlate (Biolog, Inc., Hayward, CA, USA) is a tool recently developed for characterization of functional aspects (enzymatic capacity) in soil microbial communities and were used to study a subset of soil samples. Four soil samples from forests and one from an urban area were used. Clear patterns to categorize the soil microbial activity in different habitats were not detected, which is likely to be due to the low number of biological replicates. The test illustrates the apparent heterogeneity of soil as a substrate and underlines the importance of adequate number of replicates across habitats and the need to take care of standardized sampling and implementation of the method.

Key words: *Phytophthora*, *Fagus sylvatica, Quercus robur,* soils, Biolog EcoPlates, Metabolic diversity

Table of contents

List of Figures

List of Tables

Abbreviations

AWCD	Average Well Colour Development
C	Celsius
E	Evenness
Fig	Figure
FW	Fresh weight
h	hours
H’	Shannon Diversity Index
ITS	Internal Transcript Spacer
ml	millilitre
nm	nanometre
OD	Optical Density
OUT	Operational taxonomic unit
R	Richness
PCR	Polymerase Chain Reaction
RPM	Revolutions per minute
SD	Standard deviation
T	Time
Tab	Table

1. Introduction

1.1 Introduced forest pathogens – a threat to production and biodiversity

Most pathogenic effects on trees are caused by viruses, phytoplasmas, bacteria, fungi, fungal-like organisms, parasitic plants and nematodes (Gonthier & Nicolotti, 2013). Forest pathogens are ecologically important for dead wood and nutrient cycling but can also be great threats to forest ecosystems and are a major cause of timber and growth loss (Edmonds, 2013). The degree of virulence of the pathogen, susceptibility of the plant and a favourable environment for the pathogen determine the severity of the disease (Agrios, 2005). Host plants that have evolved with a pathogen have developed defence mechanisms and in these cases the pathogen's impacts are negligible. If, however, a pathogen is introduced to another environment it may suddenly become destructive and invasive (Brasier, 2008; Edmonds, 2013). The management of these alien pathogens is often challenging because of limited knowledge of the interaction between the pathogen and the host plant (Pautasso, 2013). Some of the most devastating invasive forest pathogens belong to the genus *Phytophthora*. Two common and aggressive species are *Phytophthora cinnamomi* (RANDS), destroying many wooden species in warm environments, and *Phytophthora ramorum* (WERRES, DE COCK & MAN IN'T VELD), well known through the sudden oak death. Both pathogens are generalists which makes them very successful (Oliva et al., 2013; Hansen, 2015).

1.2.1 *Phytophthora* species – biology and impacts

Phytophthora is an oomycete genus belonging to the kingdom Straminopiles, making them related to diatoms and seaweeds (Thines, 2014). The name *Phytophthora* derives from the Greek words φυτόν (phytón), "plant" and φθορά (phthorá) "destruction", which means "the plant-destroyer". Estimations show that *Phytophthora* is responsible for more than 66 % of all fine root diseases and for 90 % of all collar rots of woody plants (Jung et al., 2016). Most famous is *Phytophthora* for its role in the Irish potato famine (Ribeiro, 2013). In Europe important broadleaf species, as European beech (*Fagus sylvatica* L.) and *Quercus* spp. are threatened by several *Phytophthora* species (Jung et al., 2013b). With more than a hundred known species and continuous discovery of new species, *Phytophthora* is a massive threat to crops and ecosystems (Kroon et al., 2012).

Phytophthora species can grow filamentously and spread through spores like fungi (Hansen, 2015). Nevertheless, Oomycetes differ from true fungi in several ways. For instance, they are diploid and have cellulose as major glucan instead of chitin in their cell walls (Bartnicki-Garcia, 1968; Judelson & Blanco, 2005). However, the main reason for the distinction is that *Phytophthora* species produce zoospores, dispersal spores that are mobile in water because they are equipped with two flagella, which is a key feature of the kingdom Straminopiles (Dick, 2001). Zoospores are asexual spores released from specific, spore-forming enclosures called sporangia, and are very successful in colonizing new plants because of their movability (Judelson & Blanco, 2005). By rapidly moving through water and moist soils, they can reach new hosts (Hansen, 2015). Zoospores detect the host tissue through chemotaxis to often non-specific root exudates (Tyler, 2001). Afterwards the zoospores encyst, immediately germinate and hyphae start growing intercellular and later intracellular into the plant (Judelson & Blanco, 2005; Oßwald et al., 2014). While the zoospores only live for a short time, *Phytophthora* species also produce resting spores, sexual oospores and asexual chlamydospores, which in contrast have thick cell walls with a chemical composition allowing them to endure microbial attacks and unfavourable conditions for several years (Judelson & Blanco, 2005; Jung et al., 2013a). Most *Phytophthora* species are soilborne and they commonly infect a host through fine roots (Oßwald et al., 2014). *Phytophthora* is hemibiotrophic, meaning that they are parasites on living tissue in the beginning and continue to utilize the dead tissue (Oßwald et al., 2014) Because of the complex biology and good adaptation to life as a plant pathogen, the control and management of *Phytophthora* diseases is a challenging task.

Since the 1990s, the number of introduced alien oomycetes in Europe has tripled and these pathogens cause increasing harm on several important broadleaf species (Jung et al., 2013b; Satini et al.,2013). Most important declines and diebacks caused by *Phytophthora* in Europe are beech decline, oak decline, root and collar rot epidemic of alders and ink disease of chestnut (*Castanea sativa* MILL) (Jung et al., 2013b). The first symptoms are usually crown dieback, yellowish foliage and deterioration of crown structure through stunted growth which indicates problems with the fine root system. Further signs are bleeding cankers at the outer and orange-brown necrosis of the inner bark (Jung, 2009) (Fig. 1). Infestation is often triggered by external conditions such as excessive rainfalls and flooding as well as droughts (Jung, 2009).

Figure 1: Symptoms of *Phytophthora* infection on European beech. Photo: Johanna Witzell

During the past years, *Phytophthora* species which are causing declines and diebacks have also been increasingly found in Southern Swedish broadleaf trees and forest stands of European beech and pedunculate oak (*Quercus robur* L.) (Jönsson, et al. 2003; Jung et al., 2013b) as well as in nurseries (Redondo et al., 2018). In Sweden *Phytophthora quercina* (TJUNG), *Phytophthora cactorum* (LEBERT & COHN) J. SCHRÖT and *Phytophthora cambivora* (PETRI) BUISMAN could be found in declining Pedunculate oak stands. A study in Southern Sweden shows that oak stands with *P. quercina* in the soil are more likely to decline (Jönsson et al., 2005). Furthermore, *P. cactorum, P. cambivora, Phytophthora gonapodyides* (PETERSEN) BUISMAN*, Phytophthora plurivora* (JUNG & BURGESS) and *Phytophthora* syringae (KLEBAHN) were found in beech dominated urban forest setting in southern Skåne (Blomquist, 2016). Further experiments have shown that *P. cactorum* and *P. cambivora* and *P. plurivora* can cause stem lesions on inoculated beech and oak seedlings (Cleary et al., 2016). However, little is still known about the pathways of their spreading and factors affecting their establishment in forests.

1.2.2 Pathways of spreading

Through intensified international trade and transports, the risk of spreading invasive *Phytophthora* species has increased because infected plants and pathogen contaminated soils are moved at an unprecedented rate and on global scale. Furthermore, climate change may have an influence on the interaction between hosts and pathogens (La Porta et al., 2008), possibly facilitating establishments of thermophilic *Phytophthora* species also in the northern parts of Europe. Pathogens may also naturally extend their range in the new climate, and host plants may become more susceptible due to abiotic stress (Sturrock et al., 2011). From 14th of December 2019 most plants need a phytosanitary certificate to enter the European Union (EU) and a plant passport for travel within the EU (Regulation (EU) 2016/2031). However, pathogens easily escape the border controls, because infections are not easily detected. In the spreading of *Phytophthora*, nurseries have been suggested to have a key position (Jung et al. 2016). Numerous nurseries in central Europe have been found to be infested by several *Phytophthora* species such as *P. cactorum* and *P. cambivorum* (Jung, 2009; Santini et al., 2013; Jung et al. 2015). Frequent planting of ornamental trees from large international nurseries in gardens, urban forests and parks increases the risk of spread in urban areas. From there *Phytophthora* can be spread by humans, animals or water into natural forests (Redondo et al., 2018). Several *Phytophthora* species that cause damage on forest trees are soilborne, which makes soil an important pathway for the spread of *Phytophthora* (Oßwald et al., 2014). By collecting soil and leaf litter form walkers' boots it was shown that over 30 % of the samples were infected with *Phytophthora* (Webber & Rose, 2008). Infestation of commercial soils could be an important pathway for the spread of *Phytophthora*, for instance because ornamental plants are sold with the planting soil. It is common to throw garden

debris into the forest, and the usage of infested soil in nurseries, followed up by planting infested seedlings in the forest may also be a potential pathway of spreading. However, we know little about the presence of *Phytophthora* infestation in commercial soils.

Soil is, however, not only interesting as a medium for the spread of pathogens but also in terms of the potential of soil microorganisms in promoting tree health and supressing the pathogens (Schlatter et al., 2017). Previous studies showed that microorganisms potentially can reduce the impact of *Phytophthora* on host plants (Aravind et al., 2008; Agusti et al., 2011; Syed-Ab-Rahman et al., 2018).

1.3 Methods to study soil microbiome

1.3.1 Molecular methods

For the analysis of microbial soil populations, soil microorganisms were traditionally cultivated on agar and the isolated strains from cultures were used for further analyses. However, with this method it is not possible to cover the whole complex soil microbiome from an environmental sample because it lacks the diversity of non-culturable microbiota (van Elsas & Boersma, 2010). Direct DNA extraction and high throughput sequencing has therefore improved the ability of performing community structure examinations of soil organisms. However, the methods for sequencing oomycetes are still limited compared to fungi and bacteria (Sapoka & Nicolaisen, 2015). Further difficulties are due to the heterogeneity of environmental samples. Different methods and primer pairs have to be used for targeting different taxa (Lear et al., 2017). Yet, even though high throughput sequencing gives advantages in community species composition, it still does not inform about ecological functions of the microbiomes or individual species within it.

1.3.2 Functional analysis of soil microbiome

Plate counting is the traditional method to study microbial diversity. However, it is limited by its ability to produce large quantity of cultures. Using sole carbon source utilization on the other hand enables a highly reproducible way, generating a large amount of data (Kirk et al. 2004). Biolog EcoPlate (Biolog, Inc., Hayward, CA, USA) is one of the tools created for evaluation of changes in functional microbial communities in microbial ecology studies based on sole carbon source utilization. The tool is especially designed for analysis of catabolic enzymatic capacity, an important functional trait of microbes in environmental samples (Preston-Mafham et al., 2002). The method is based on a 96-well microtiter plate, loaded with 31 different carbon sources and one blank well, each with three replications (Appendix 1). Nine of those carbon sources are known components of root exudates (Preston-Mafham

et al., 2002). By inoculating environmental samples on those carbon sources, an analysis of the enzymatic capacity of the microbial community to utilize these substances can be performed. The utilization of substances can be measured by the colour development of each well. Through the determination of metabolic diversity, it is possible to create a metabolic finger print for the studied communities (Gryta et al., 2014). This metabolic finger print helps to understand ecological and ecosystem functions of the microbiomes (Miki et al., 2016). For instance, it could be possible to understand, if some communities are active in utilizing plant root exudates such as 2-Hydroxy Benzoic Acid, 4-Hydroxy Benzoic Acid, D-Malic Acid. D-Xylose, L-Arginine, L-Asparagine, L-Serine, L-Threonine and alpha-D-Lactose (Campbell et al., 1997). Root exudes may have a regulating effect on soil microbial communities by either supporting beneficial symbioses or as antimicrobial defence secretion (Walker et al., 2003). In the long term, when information about occurrence of Phytophthora in different soils increases, Biolog EcoPlate could also be a tool to characterize forest soils with different capacities to suppress tree-pathogenic *Phytophthora* species, but to my knowledge, this has not yet been tested.

1.4 Aim of the study

Knowledge about present pathogenic *Phytophthora* species in substrates and landscapes are essential to counteract the further spread of these pathogens in our nature. The goal of this study was to add to the current knowledge about the pathways of spreading for *Phytophthora* pathogens in our nature, by analysing different soils, including commercially available ones, for the presence of *Phytophthora*. In addition, the study aimed to test the Biolog EcoPlate as a tool to characterize the microbial communities in heterogeneous soil samples.

2. Material & Method

2.1 Soil samples

Three different types of soil samples were used in the analyses. Five soil samples derived from commercially available planting soils, purchased from plant retailers (Tab. 1). One bag of commercial soil was considered as one sample. Five different products of two common brands were used. In addition, soil samples were collected from five urban and eight forest stands in Halland county and in Gothenburg during the period from November 2018 to February 2019 (Fig. 1). These stands were selected based on suspicion of *Phytophthora* infestation (crown dieback, bleeding lesions on trees). Eight samples were taken from soil from forests dominated by *Fagus sylvatica* and five soil samples from forests dominated by *Quercus robur*.

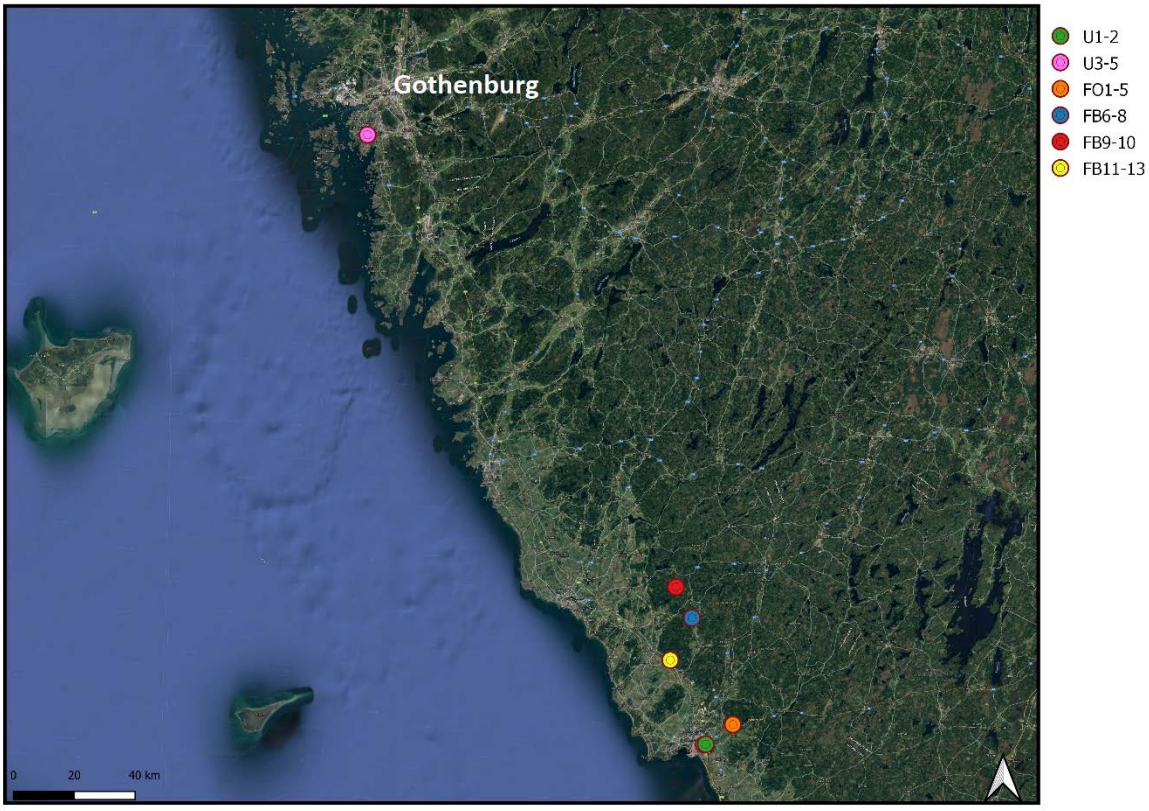

Figure 2: Sampling areas in Halland county and Gothenburg, urban soil (U), oak forest (FO) and beech forest (FB)

Table 1: Information about soil samples

ID	Date	Soil type	Tree species
C1	-	Commercial	-
C2	-	Commercial	-
C3	-	Commercial	-
C4	-	Commercial	-
C5	-	Commercial	-
U1	18.11.2018	Urban	Beech
U2	18.11.2018	Urban	Beech
U3	06.01.2019	Urban	Beech
U4	06.01.2019	Urban	Beech
U5	06.01.2019	Urban	Beech
FO1	06.02.2019	Forest	Oak
FO2	06.02.2019	Forest	Oak
FO3	06.02.2019	Forest	Oak
FO4	06.02.2019	Forest	Oak
FO5	06.02.2019	Forest	Oak
FB6	08.11.2018	Forest	Beech
FB7	08.11.2018	Forest	Beech
FB8	08.11.2018	Forest	Beech
FB9	08.11.2018	Forest	Beech
FB10	08.11.2018	Forest	Beech
FB11	08.11.2018	Forest	Beech
FB12	08.11.2018	Forest	Beech
FB13	08.11.2018	Forest	Beech

In forests and urban areas, soil samples were obtained from four sides around a tree with suspected *Phytophthora* infection. The position was recorded by GPS (Garmin eTrex Legend® Cx). To collect the soil a garden spade and a hand shovel were used. The four soil samples were pooled to one composite sample. To avoid cross-contamination tools were cleaned with a brush and by spraying with 70 % ethanol solution before each sampling. The soil was then transported in sealed plastic bags to the laboratory where it was stored in the freezer.

2.2 Laboratory analyses

2.2.1 DNA analysis to detect *Phytophthora*

Sub-samples of each soil sample were transferred into 50 ml centrifuge tubes and freeze dried. The DNeasy PowerMax Soil Kit was used for isolating the microbial DNA from the soil. The isolation was made according to the Quick-Start Protocol (2016) (Appendix 2). Afterwards the samples were sent to MR DNA (www.mrdnalab.com, Shallowater, TX, USA)) for polymerase chain reaction (PCR) and sequencing. The internal transcript spacers (ITS) ITS1oo and ITS4 were used as primers in a 33 cycle Polymerase Chain Reaction (PCR) using a HotStarTaq Plus Master Mix Kit (Qiagen, USA). ITS1oo is an oomycete specific primer which showed improved specificity for oomycetes in previous studies when paired with the universal primer ITS4 (Riit et al., 2016). Amplification was proceeded under following conditions: Denaturation at 94°C for 3 minutes, followed by 33 cycles of 94°C for 30 seconds, annealing at 53°C for 40 seconds and extension step at 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. The sequencing was performed using PacBio 5kb Kit following the manufacturer's guideline. The samples C2 and FB11 were not analysed.

2.3.2 Soil microbial community profiling using Biolog EcoPlates

Four soil samples with forest origin (FO1, FO2, FB6, FB12) and one sample with urban origin (U1) were used for the test of microbial community profiling. For each soil sample three technical replicates were prepared. A soil suspension was created for the soil samples as follows: Five FW grams of soil was suspended to 50 ml of autoclaved ultra pure water. The suspension was agitated in an orbital shaker (160 RPM) for one hour. Afterwards the suspension was allowed to settle for 30 minutes at 4°C. One ml soil suspension was diluted with 100 ml of autoclaved water. Of the diluted sample, 100 µl were pipetted into each well of the Biolog EcoPlate. Subsequently the wells were immediately measured with a microplate reader (BMG LABTECH SPECTROstar® Nano) to obtain values at t=0. Absorbance was measured at 590nm where the peak absorbance of tetrazolium dye occurs (Zack et al. 1994). Measurements were taken daily (24 h intervals) for four days. The incubation time was determined by a previous trial, where the highest utilization could be observed after 96 hours of incubation. Between measurements, a lid was placed on each plate which was sealed with parafilm to avoid evaporation, and the plates were incubated at 25°C in darkness. Obtained results were converted into excel spreadsheets.

2.4 Data analysis

2.4.1 Identification of sequences

Operational taxonomic units (OTUs) were generated and defined by clustering at 3 % divergence. The final OTUs were taxonomically classified by BLASTn against a database derived from RDPII http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov). The sequences were visualized by using Bioedit (3.3.19.0). For verification of the results, the sequences of the OTUs were copied as Fasta format and BLASTn was used at the reference database at the National Center for Biotechnology Informatics (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

2.4.2 Functional analysis of soil microbiome

The average absorbance value of the control wells was subtracted from each well containing a carbon source to create the net absorbance value. The net absorbance value gives a corrected optical density (OD) value. In case of negative reading the net absorbance value was set to 0. (Blumenstein, 2015). To calculate the average well colour development (AWCD) following formula was used:

$$AWCD=\Sigma\ ODi/31$$

To analyse the capability of the microbial communities to utilize different carbon sources, the sources were sorted into carbon groups according to Chazarenc et al. (2010) (Tab. 2) and analysed at 96 h where the highest utilization was reached. In addition, utilization patterns of some individual compounds (carboxylic acids: 2-Hydroxy Benzoic Acid, 4-Hydroxy Benzoic Acids, D-Malic Acid; amino acids: L-Arginine, L-Asparagine, L-Serine, L-Threonine; Carbohydrate: D-Xylose, alpha-D-Lactose) with specific importance as root exudates were examined.

Table 2: Carbon sources on EcoPlates reproduced after Chazarenc et al. (2010).

Amines	Amino acids	Carbohydrates	Carboxylic Acids	Complex Carbon Sources	Phosphate-carbon
Phenylethylamine	L-Arginine	β-Methyl-D-Glucoside	D-Galactonic Acid γ Lactone	Tween 40	Glucose-1-Phosphate
Putrescine	L-asparagine	D-Xylose	Pyruvic Acid Methyl Ester	Tween 80	D-L-α-Glycerol Phosphate
	L-Phenylatanine	i-Erythritol	D-Galacturonic Acid	α-Cyclodextrin	
	L-Serine	D-Mannitol	2-Hydroxy Benzoic Acid	Glycogen	
	L-Threonine	N-Acetyl-D-Glucosamine	4-Hydroxy Benzoic Acid		
	Glycyl-L-Glutamic Acid	D-Cellobiose	γ-Hydroxybutyric Acid		
		α-D-Lactose	D-Glucosominic Acid		
			Itaconic Acid		
			α-Ketobutyric Acid		
			D-Malic Acid		

The substrate use richness was measured by summing the number of positive responses. A threshold was set at OD= 0,1784 to indicate carbon utilization. The threshold was created by the average absorbance values of oxidized substrates as defined by Vahjen et al. (1997).

Shannon diversity index was used to analyse substrate richness and substrates evenness:

$$H' = -\Sigma pi(\ln pi)$$

where p is the proportional microbial activity on substrate *i* divided by total microbial activity (Stefanowicz, 2006; Zak et al., 1994).

Shannon evenness which measurers the uniformity of activities across the utilized substrates:

$$E = H/\text{Ln } S$$

where H is the Shannon diversity and S the richness of substrate utilization (Zak et al. (1994).

The data was analysed and displayed by using JMP Pro 14.

3 Results

3.1 DNA analysis

Several OTUs attributed to *Phytophthora* species were found. However, for most species the maximum score and maximum identity values were too low to identify the species with certainty. Only *P. cambivora and P. citricola*, which has been renamed *P. plurivora* (Jung & Burgess, 2009), could be attributed with high likelihood because of an e-value of 0 to several OTUs (Appendix 3). Nine OTUs related to *P. cambivora* were found in all soil samples from oak forests and in all commercial soil samples. Furthermore, they were present in all soil samples but one from beech forests and urban areas. The OTU attributed to the *P. plurivora* was only found in U1. FB8 was the sample with most identified OTUs (Fig. 3).

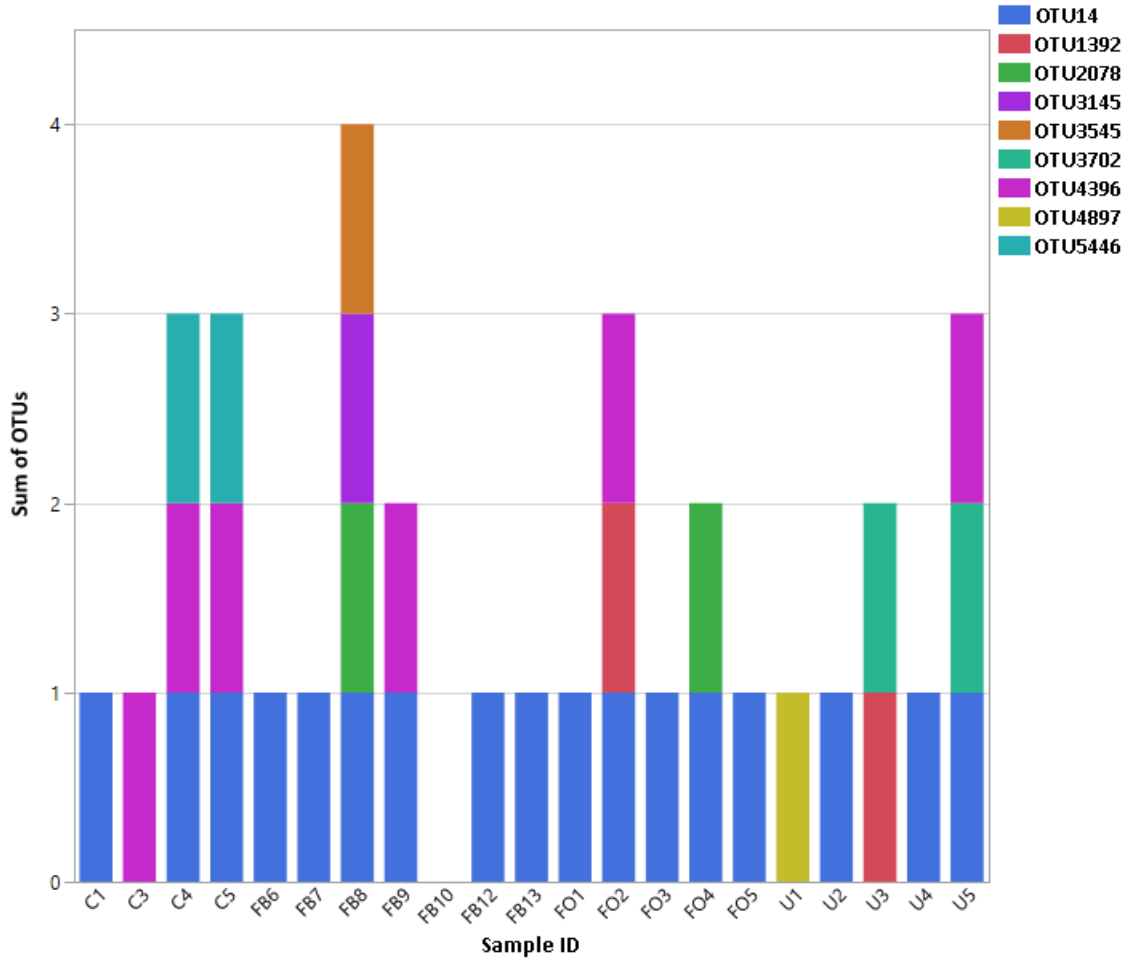

Figure 3: Operational taxonomic units (OTU) identified on *Phytophthora* species grouped by soil samples. All OTUs were classified as *P. cambivora*, except for OTU 4897 which was classified as *P. plurivora*. Samples are taken from commercial soils (C), Urban soil (U), oak forest (FO) and beech forest (FB)

Looking at the clusters of organisms OTU 14 was by far the most common across the samples, followed by OTU 4396. Four OTUs were only present in one sample (Fig. 4).

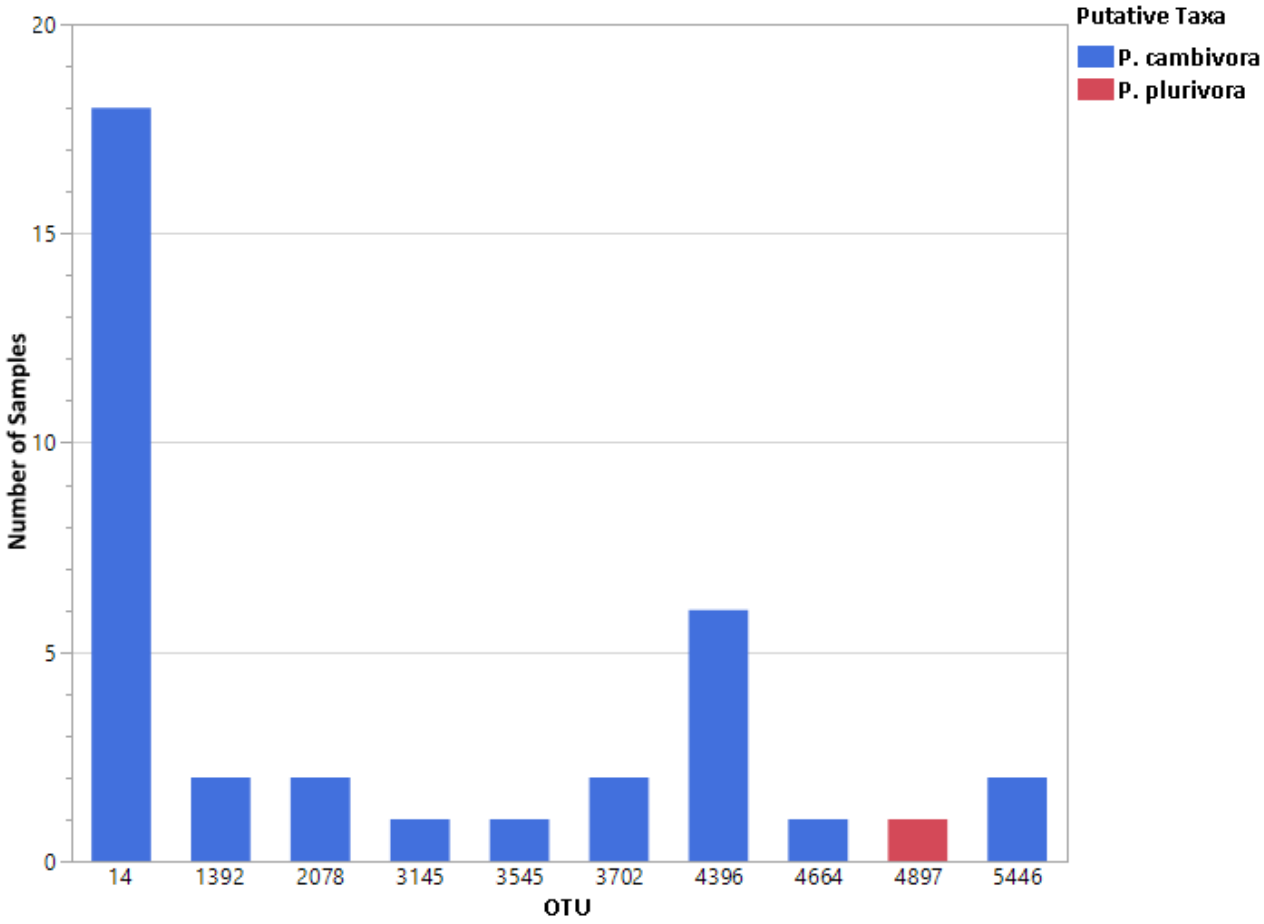

Figure 4: Number of samples with OTUs attributed to *Phytophthora cambivora* and *P. plurivora*

3.2 Soil microbial community profiling

The temporal dynamics in AWCD differed between samples, with FO2 (forest, oak) and U1 (urban, beech) having the highest temporal changes (Fig. 5). All samples reached their highest AWCD after 96 h of incubation wherefore the AWCD (96h) value was used for analysis of utilization of different carbon groups (Fig. 6). FB12 (forest, beech) had the lowest OD_{590}. The AWCD followed different patterns, FO2 and U1 follow both following a sigmoidal-curve. The other samples all had their own pattern with incubation time. The samples from oak forest FO1 and FO2 had more differentiated patterns than the samples from beech forest FB6 and FB12

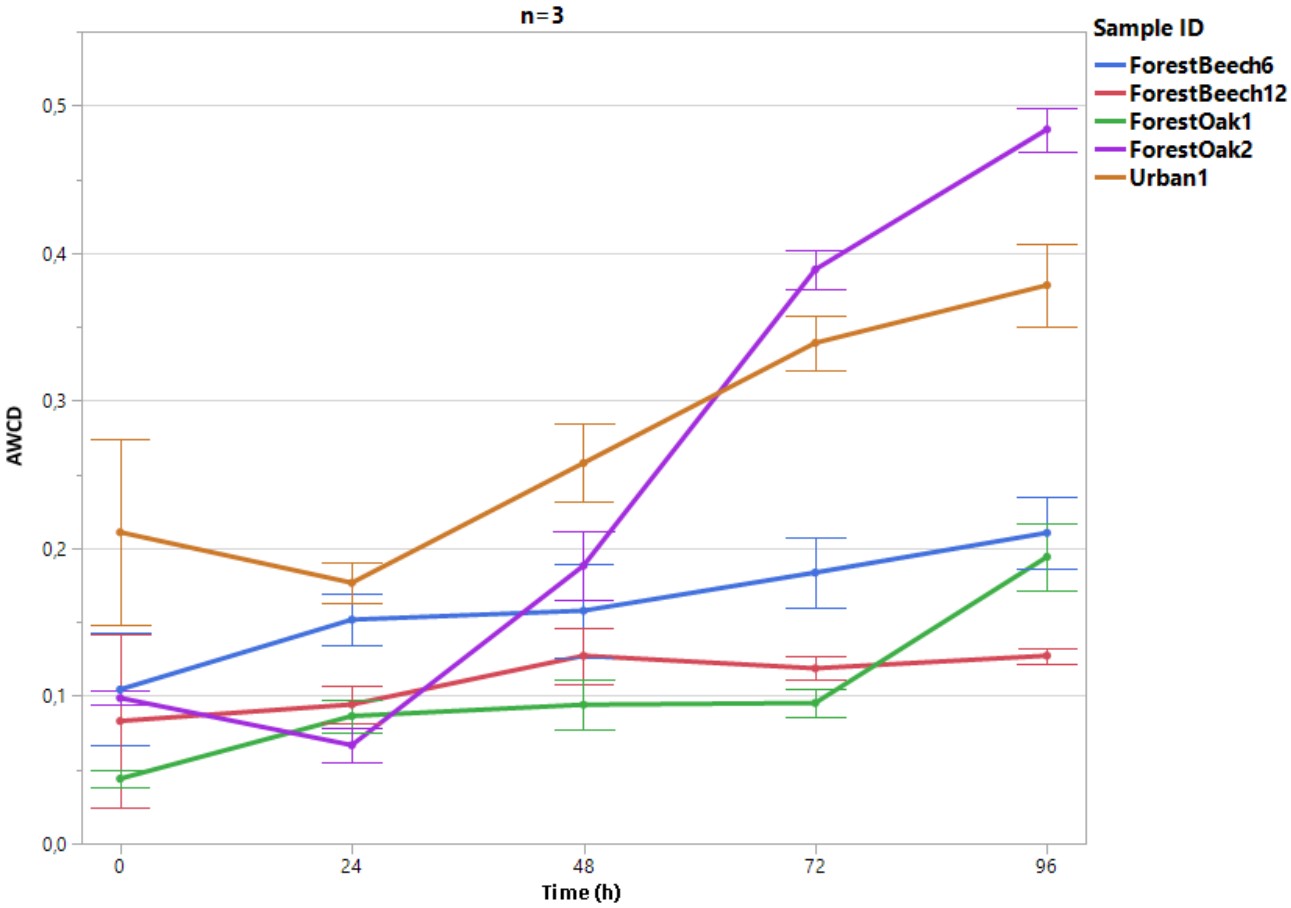

Figure 5: Mean values of average well colour development (AWCD) of five different samples (three technical replicates) on Biolog EcoPlates based on 96 h of incubation. Soil samples are obtained from oak forest, beech forest and one soil sample from an urban area.

The microbial communities were able to utilize all types of carbon sources. Amines, amino acids, carbohydrates and carboxylic acids had similar patterns of utilization across the samples with the highest utilization in FO2 and U1, and the lowest in FB12. Complex carbon sources were most actively utilized by microbes in U1. Phosphate carbon sources were almost equally utilized across all samples with a rather low utilization in total (Fig. 6).

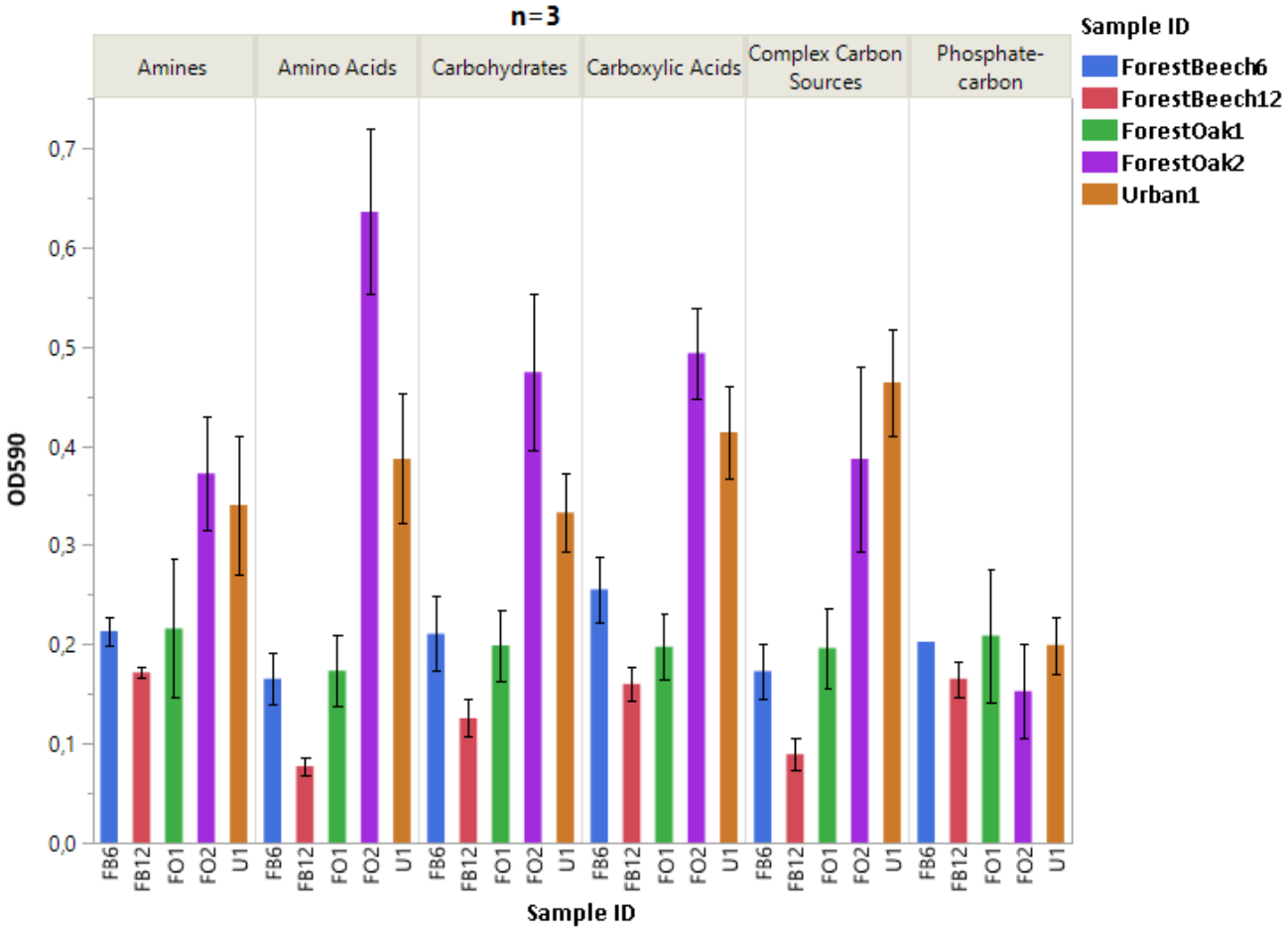

Figure 6: Mean utilization and SD (three technical replicates) of different carbon groups by soil sample microbes based on 96-h incubation measured as OD590 nm. Soil samples are obtained from oak forest, beech forest and one urban area.

The amino acids L-Arginine, L-Asparagine and L-Serine had similar patterns with highest utilisation by FO2. The utilization of those thee amino acids seems to be higher in FO2 and U1 than the mean utilization of amino acids after 96 hours of incubation. In U4, 4-Hydroxy Benzoic Acid seems to be more utilized than other carboxylic acids (Fig. 7).

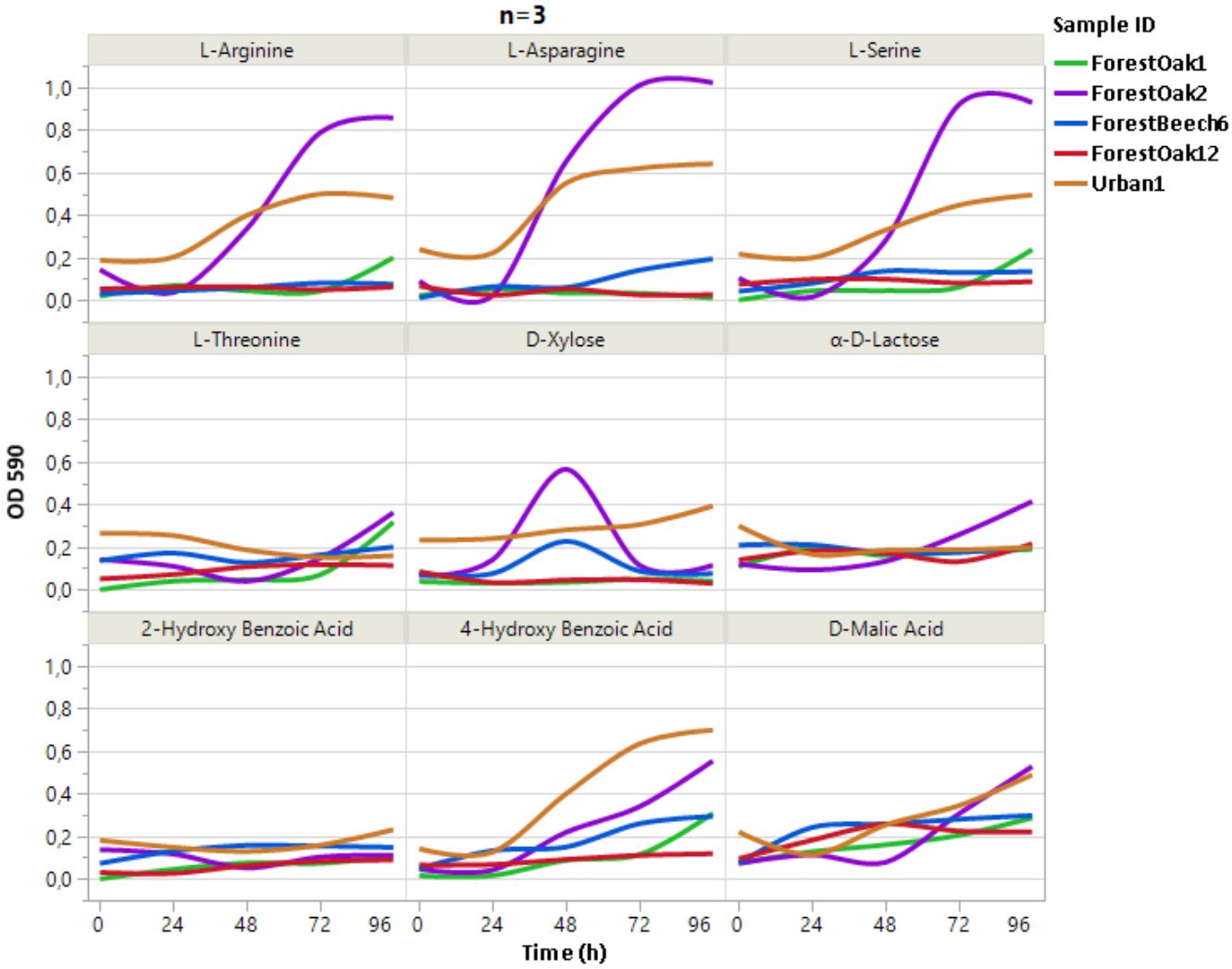

Figure 7: Average well colour development (AWCD) of nine carbon sources from three different carbon groups related to plant root exudes. Five samples (three technical replicates) were used. Soil samples are obtained from oak forest, beech forest and one soil sample from an urban area.

In all samples, the number of utilized substrates increased during incubation time and the richest carbon source utilization was observed after 96 h of incubation. The sample U1 had the richest utilization overall at any given time point. All samples, except FO1 and FB12, had over 50 % of the carbon sources utilized after 96 hours of incubation. (Fig. 8).

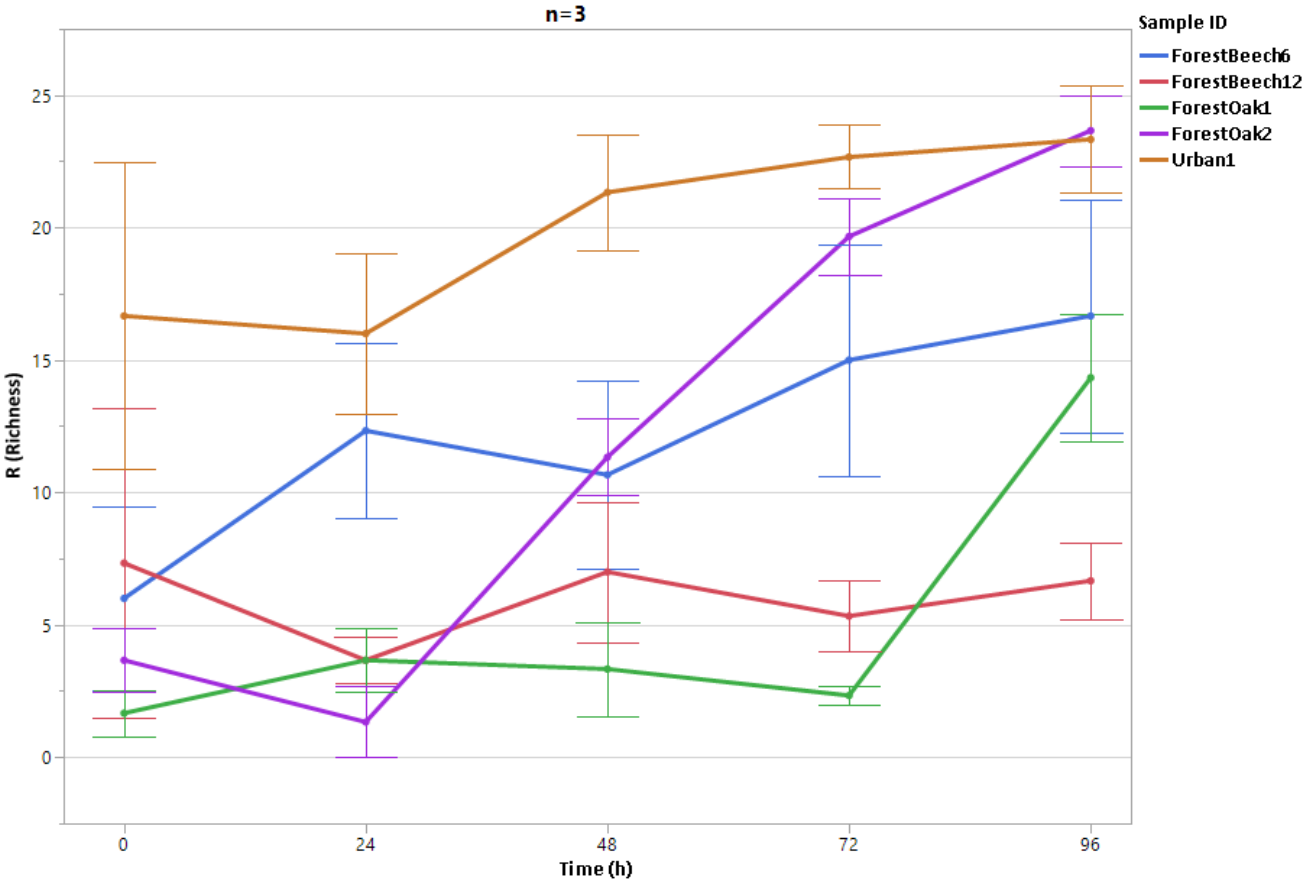

Figure 8: Number of utilized substrates by different samples showing the mean number of carbon sources (three technical replicates) for which the optical density was at least 0,1784. Soil samples were obtained from oak forest, beech forest and one urban area.

Sample U1 had the highest substrate utilization diversity. The patterns of H' are similar to the AWCD for each sample. However, diversity differs to AWCD by U1 seemingly having a higher diversity than FO2 after 96 h of incubation. (Fig. 9).

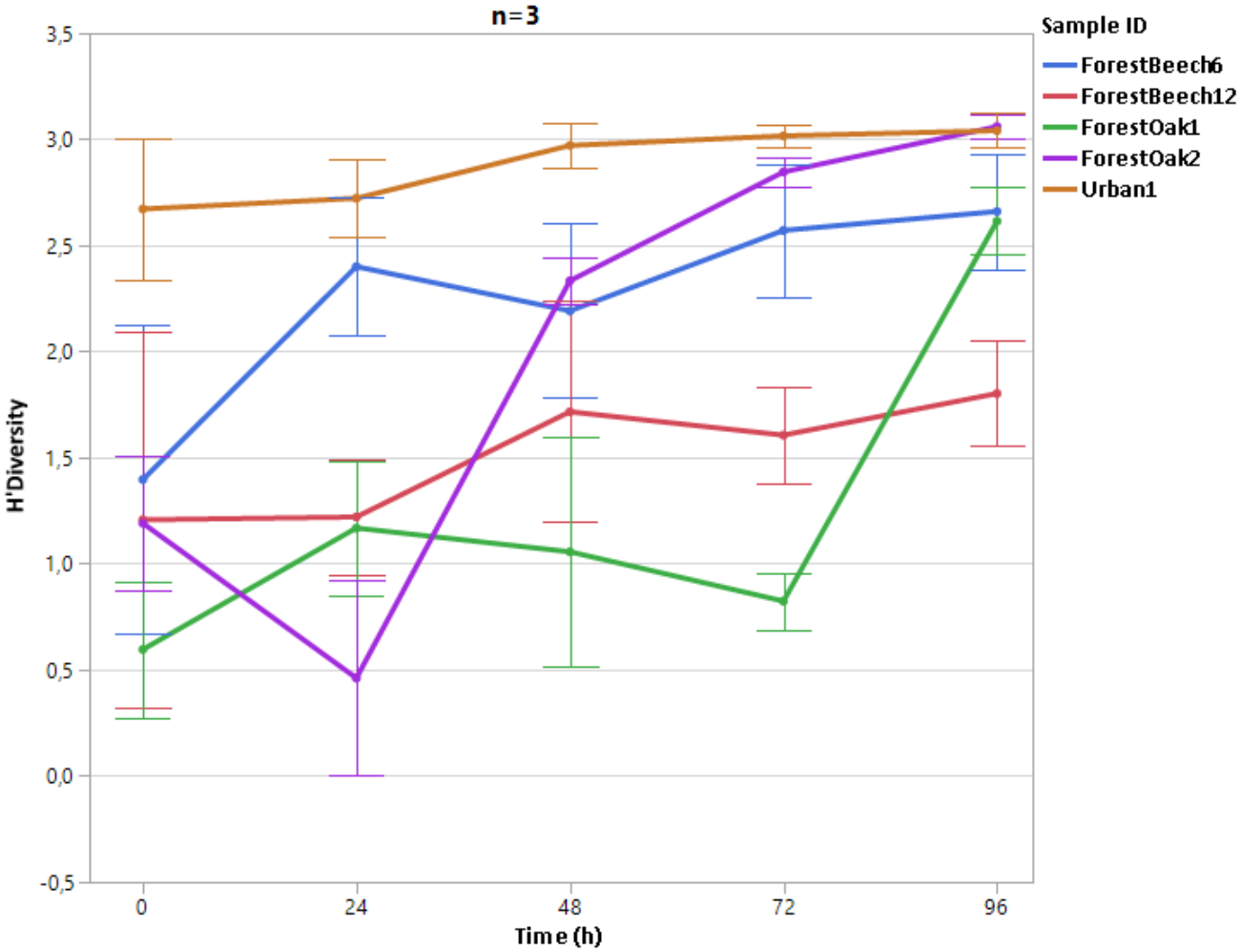

Figure 9: Shannon diversity patterns from different samples (three technical replicate) on Biolog EcoPlate. Soil samples were obtained from oak forest, beech forest and one soil sample from an urban area.

Evenness (E) was calculated when enough activity was present. In case Richness (R) are was 0 or 1 no calculation of evenness was possible. The value of E was constantly over 0,9 for all sample, when calculation was possible (Fig. 10).

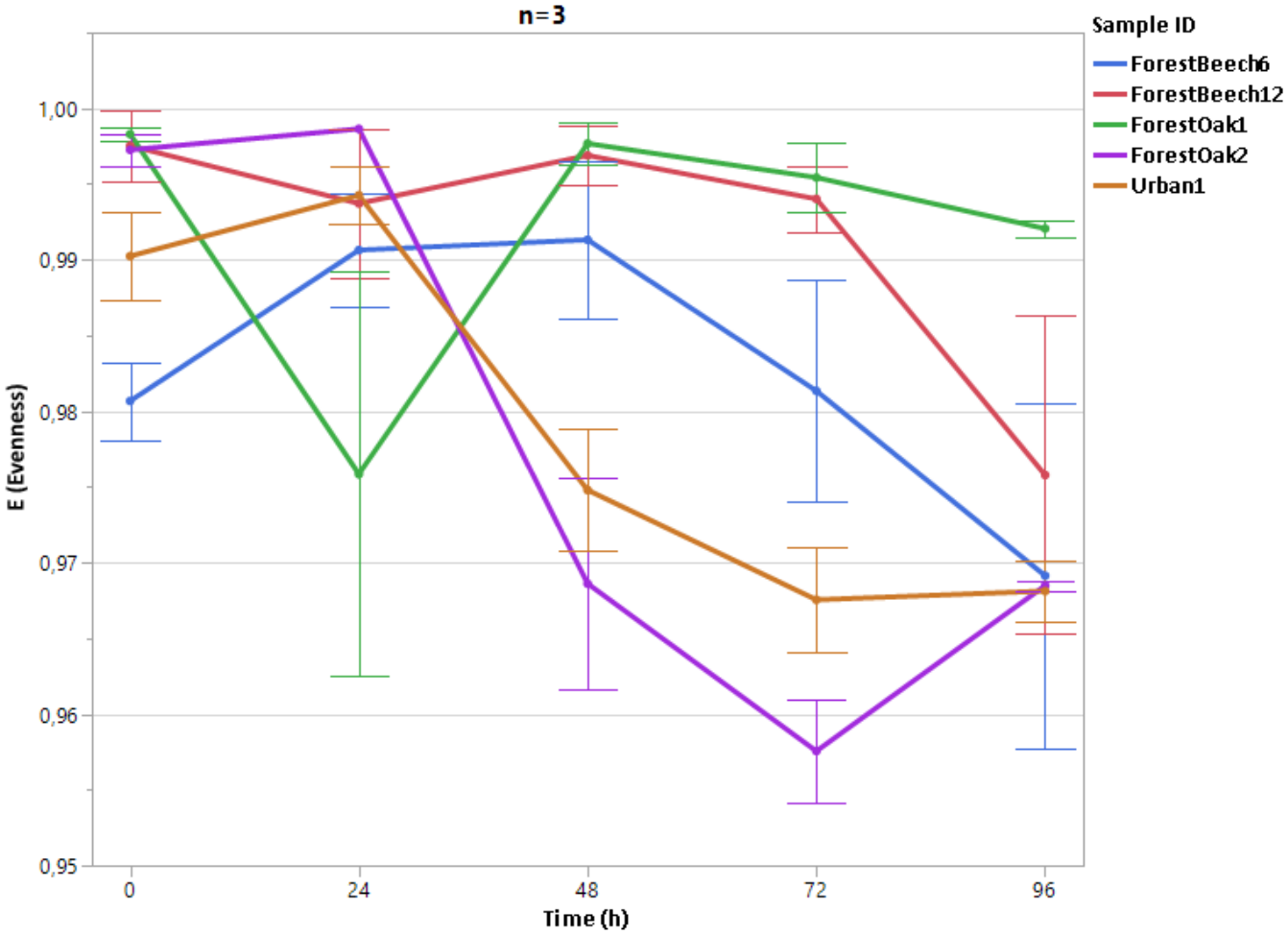

Figure 10: Pattern of evenness (E) of substrates utilization of different samples (three technical replicates). Samples were obtained from oak forest, beech forest and one an urban area.

A compilation of results at 96 h of incubation can be found in Appendix 4.

4. Discussion

4.1 Occurrence of *Phytophthora* in soil samples

The DNA analysis of soil samples confirmed the presence of two *Phytophthora* species, *P. cambivora* and *P. citricola* which is today called *Phytophthora plurivora* (Jung & Burgess, 2009). Both species are known to cause bleeding cankers in beech and oak, and they are considered as the most common aggressive *Phytophthora* pathogens affecting European beech (Jung et al., 2013b). The finding that *P. cambivora* was found in almost all samples, in forest and urban landscapes as well as in commercial soils is alarming and suggests that *P. cambivora* can effectively spread across different habitats in Sweden. Intriguingly, however, *P. plurivora* was only detected in one sample from the urban area in Halmstad. Thus, the commonness of *P. plurivora* in different habitats was not confirmed, which contrasts with earlier studies where this species has been found in nurseries, urban and natural forests in Southern Sweden (Redondo et al, 2015; Redondo et al., 2018).

Phytophthora cinnamomi could be verified by using BLASTn on the reference databases of NCBI, however the e value and total score were too low to confirm the species. *P. cinnamomi* has not been found in in Swedish forests so far which could be explained by the species' restriction to warmer climates (Brasier, 1995; Brasier & Jung, 2006). However, it has been detected in samples of ornamental plants in Sweden (M. Cleary, unpublished data), and in future climate it could also become a problem in Northern Europe.

Clear patterns in *Phytophthora* occurrence or the abundance of different operational taxonomic units between oak and beech samples, or forest and urban samples were not detected. This may be because the sampling effort in my study was limited due to practical reasons, and the material thus does not allow rigorous statistical comparisons. However, the lack of clear patterns may also be indicative for the commonness of several *Phytophthora* species in the region. Recent studies have indicated that several *Phytophthora* species, including *P. cambivora* and *P. plurivora,* have already spread across different environments. In a study by Redondo et al. (2018) *P. cambivora* was frequently found in forests but was not one of the most frequent *Phytophthora* species in nurseries which could indicate that it is already established in the region. Blomquist (2017) identified five different *Phytophthora* species in beech forests and Jönssson (2003) isolated three *Phytophthora* species from oak stands. Both studies were conducted in Southern Sweden and *P. cambivora* was one of the identified species, which substantiates the wide spread across habitats in the region. The high susceptibility of Swedish tree species to *Phytophthora* was indicated in a study by Cleary et al. (2016) where *P. cactorum*, *P.*

cambivora and *P. plurivora* were causing lesions of different degree on all tested tree species. This susceptibility of Swedish tree species can be seen as a factor that allows *Phytophthora* species to spread across different environments.

It is assumed that *Phytophthora* species are introduced through plantings using imported nursery materials and there is already proof of many infestations of *Phytophthora* species across European nurseries. Jung et al. (2015) found *Phytophthora* infestation in 91,5 % of examined nurseries in Europe. The finding of *P. cambivora* in commercial soils from convenient stores verifies another potential pathway for spreading. By using commercial soils in garden or by littering garden debris in forest edges or forests, citizens could assist the introduction of *Phytophthora* into ecosystems. This finding emphasizes the importance and the need to educate people about the danger of spreading pathogens in forests through gardening or careless deposition of plant residues.

The spread of *Phytophthora* species is dependent on asexual motile zoospore (Judelson & Blanco, 2005). The study by Redondo et al. (2018) has shown that the proportion of *Phytophthora* species with asexual structures was significantly higher in forests than in nurseries, indicating the need of zoospores for establishment. In the same study it was indicated that *Phytophthora* species with lower cardinal temperature were more likely to be found in forests. With likely rising temperatures in the future *Phytophthora* species may find more suitable conditions and expand. It is important to hinder further expansion and to recognize potential risks early by monitoring non-native *Phytophthora* species. Considering the important role of zoospores for spreading, it would be reasonable to develop control methods to counter the zoospores before the species establishes, because zoospores are more vulnerable than resting spores (Judelson & Blanco, 2005).

4.1.2 Limitations of the analysis

Despite having many OTUs related to Phytophthora, there were only two species which could be determined to species level: the putative taxa could not always be validated by BLASTn on the NCBI database. This may be due to the chosen ITS primers ITS1oo and ITS4 as they have a higher specificity to oomycetes and better taxon coverage compared to other primer pairs. Furthermore, the PacBio allows a longer read length compared to other platforms. Therefore, the sequence read in NCBI database may cover other ITS regions and/or uses shorter reads wherefore it is not possible to get a perfect match or any match at all. Hence, the analysis is limited by the content of the database, and in most cases the data output (Appendix 3) should be considered merely as indicators of what genus might be present in the samples.

4.2 Soil microbial community profiling

EcoPlate results indicated that the metabolic activity of soils differed even within same habitats. This is not unexpected since soil is known to be a highly heterogenous substrate, where the microbial community structure is likely to vary in time and place (Piotrowska-Długosz et al, 2019). The microbial activity in the urban soil sample seemed to be generally higher than in the forest soil samples, which could be indicate a more diverse community in urban areas where soil is moved around and mixed due to building activities. However, the small sample number per habitat in my study does not allow to make conclusions about the habitat specific differences in the functional traits of microbial communities.

Overall, the obtained OD values were rather low compared to other studies (Ellis et al., 2001; Gryta et al.,2014). The lower AWCD values could be a result of a lower pH value in my samples. In the study of Gryta et al. (2014), not only the AWCD but also the pH value of the samples was higher (pH 5-11). In the study of Ellis et al. (2001) the soil samples with a pH value around 5 had a lower AWCD than the samples with a pH value around 7. All samples from my study were collected from broadleaf stands, where the main species were either European beech or Pedunculate oak. These tree species are known to lead to a lower pH value in soil than other broadleaf species in the region, having an approximate pH value between 4,37 and 4,53 in beech and oak forests (Oostra et al., 2006). Catabolic activity on EcoPlates is likely to be mainly conducted by fast growing bacteria though it is not known which species utilize the carbon sources (Stefanowizc, 2006). The pH has been identified as the main soil property affecting bacterial communities, generally leading to a higher bacterial diversity with a higher pH value (Kaiser et al., 2016; Rousk et al., 2010). Fungi on the other hand are more acid tolerant (Mulder et al. 2005) though they were unlikely to contribute to the colour development of the EcoPlates because they have been reported to be incapable to utilize the tetrazolium dye in these plates (Preston-Mafham et al. 2002). The high utilization in the urban sample may also result from a higher pH through input of cement, filling materials and other building debris (Räsänen & Penttala, 2004; Shukla et al., 1990). The oak forest samples FO1 and FO2 were sampled in the same area, but the AWCD differs a lot which shows that microbial communities have high heterogeneity even on small sites (Preston-Mafham et al., 2002).

The possible importance of pH for AWCD level is also implied by the result that the sample FO2 had much higher AWCD value than FO1: these samples were collected in the same area, but along a hillslope, with FO1 being collected higher up in the hill and FO2 closer to footslope in a moister soil. The slope position is known to affect the pH (Yeshaneh, 2015.) and it is possible that the movement

of water downhills may have contributed to increased pH in the footslope, thus supporting a microbe community that produced higher AWCD values. In my study the pH of the soil samples was not measured and thus the impact of it the results remain unconfirmed, but it could be recommended to add to the protocol a measurement of the pH in soil samples before the EcoPlate analyses, in order to understand the observed substrate utilisation patterns. My results also show that it is important to carefully consider that microbial communities have high heterogeneity, even within small spatial scales (Preston-Mafham et al., 2002).

Studied compound groups were utilized by the microbes in all samples, possibly reflecting the general importance of the studied substrates for soil bacteria. Interestingly, complex carbon sources were utilized at highest level in the urban sample. Since the utilized complex carbon sources, such as Tween 80, which is a surfactant used in food and different products (e.g., cosmetics), they are probably more likely to be found in an urban area than in a forest area. It is possible that microorganisms from the urban area are more adapted to using these substrates (Orwin et al., 2006).

Another noticeable result is the high utilization of amino acids by FO2, which also had the highest overall utilization. This could indicate a need for nitrogen. Interestingly, L-arginine and L-asparagine were amino acids with high utilization in FO2 and U1. Arginine and asparagine were found to be the most important amino acids in all compounds of sessile oak (Bazot et al., 2013) Under stress conditions, amino acids such as L-Asparagine can increase in the bark and wood of roots (Wargo, 1996). Furthermore, there was a high utilization in U1 of 4-Hydroxy Benzoic Acid. 4-Hydroxy Benzoic Acid, also known as p-hydroxybenzoic acid, was earlier identified as a fine root extract in European beech (Tomova et al.,2005).

A potential error in EcoPlate profiling could have been caused by the sampling during different seasons. In an earlier study by Chazarenc et al., (2010) showed in a community level physiological profiling analysis on wetlands that seasons are important parameters affecting bacterial diversity. In my study, however, the two samples with highest utilization (FO2, U1) were sampled during different seasons. Moreover, the two oak samples were collected at the same time and from the same site but showed large differences. Thus, my results indicate that other sources of variation may be more important regulators of the metabolic activity of bacterial communities.

Some technical recommendations can be compiled from my tests. For example, I found temporal dynamics in the utilization patterns and that the reaction on plates resulted in highest differences in AWCD at 96 hours incubation time, as indicated also by the fact that the highest R and H’ values were found at this time point. Thus, if point in time comparisons are made, they should not be made too

early. Different growth rate of organisms can influence the total utilization of a carbon source (Garland, 1997), wherefore the measurements should be done over an adequate amount of time. Furthermore, all samples already had a colour development at t=0. This could be due to turbidity which could be avoided by measuring a turbidity value at OD750nm and by subtracting it from the OD590 value (Classen et al., 2003).

In the future, the EcoPlate analyses could be used to compare if certain specific functional traits (catabolic activities) are linked to soils where we observe high or low risk of trees being affected by *Phytophthora*. If certain patterns of enzymatic activities are always present in soils with low occurrence of symptomatic trees, it could be studied whether the microbes causing these activities could possess traits that lead to suppression of *Phytophthora* in the soil.

5. Conclusions

Phytophthora cambivora was found in Gothenburg and Southern Halland in urban areas and forests, as well as in commercial soil samples. *P*. *plurivora* was found in Halmstad. In conclusion, these results show that tree pathogenic *Phytophthora* species are widespread in Southern Swedish landscapes and that commercial soil may be an important pathway for the spreading of these pathogens. Measures have to be taken to spread awareness about aggressive forest pathogens in commercial soils and measurements should be taken to restrict further spreading.

Biolog Ecoplate is a potential tool for studies examining the functional diversity of soil microbial communities, but it needs to be taken care to standardize the sampling and ensure adequate replication because of the spatial and time dependent variation in soil microbial communities. Further, soil properties should be measured as potential drivers of microbial community patterns. Furthermore, it is important to standardize the method to make studies comparable.

6. Acknowledgements

First, I would like to thank my supervisor Johanna Witzell for her support, advice and constructive feedback during my thesis work as well as for introducing me to laboratory work in plant pathology. I also thank Stefan Anderson, soil specialist in Forestry Agency (Halland district) for providing the beech forest soil samples. Further, I would like to thank all the people from the laboratory group who were always helpful when I had questions. The study is a part of activities in the project "*Preventing the spread of new pathogens in Nordic forests to secure sustainable forestry in growing bioeconomy*", financed by Nordic Forest Research SNS (2019–2021).

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Appendix

Appendix 1: Carbon sources in EcoPlate (source: Biolog brochure)

BIOLOG **Microbial Community Analysis**

EcoPlate™

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A5 Water	A6 β-Methyl-D-Glucoside	A7 D-Galactonic Acid γ-Lactone	A8 L-Arginine	A9 Water	A10 β-Methyl-D-Glucoside	A11 D-Galactonic Acid γ-Lactone	A12 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B5 Pyruvic Acid Methyl Ester	B6 D-Xylose	B7 D-Galacturonic Acid	B8 L-Asparagine	B9 Pyruvic Acid Methyl Ester	B10 D-Xylose	B11 D-Galacturonic Acid	B12 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C5 Tween 40	C6 i-Erythritol	C7 2-Hydroxy Benzoic Acid	C8 L-Phenylalanine	C9 Tween 40	C10 i-Erythritol	C11 2-Hydroxy Benzoic Acid	C12 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D5 Tween 80	D6 D-Mannitol	D7 4-Hydroxy Benzoic Acid	D8 L-Serine	D9 Tween 80	D10 D-Mannitol	D11 4-Hydroxy Benzoic Acid	D12 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Amino Butyric Acid	E4 L-Threonine	E5 α-Cyclodextrin	E6 N-Acetyl-D-Glucosamine	E7 γ-Amino Butyric Acid	E8 L-Threonine	E9 α-Cyclodextrin	E10 N-Acetyl-D-Glucosamine	E11 γ-Amino Butyric Acid	E12 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F5 Glycogen	F6 D-Glucosaminic Acid	F7 Itaconic Acid	F8 Glycyl-L-Glutamic Acid	F9 Glycogen	F10 D-Glucosaminic Acid	F11 Itaconic Acid	F12 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Keto Butyric Acid	G4 Phenylethyl-amine	G5 D-Cellobiose	G6 Glucose-1-Phosphate	G7 α-Keto Butyric Acid	G8 Phenylethyl-amine	G9 D-Cellobiose	G10 Glucose-1-Phosphate	G11 α-Keto Butyric Acid	G12 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H5 α-D-Lactose	H6 D,L-α-Glycerol Phosphate	H7 D-Malic Acid	H8 Putrescine	H9 α-D-Lactose	H10 D,L-α-Glycerol Phosphate	H11 D-Malic Acid	H12 Putrescine

Appendix 2: DNeasy® PowerSoil® Kit Protocol

Quick-Start Protocol June 2016

DNeasy® PowerSoil® Kit

The DNeasy PowerSoil Kit can be stored at room temperature (15–25°C) until the expiry date printed on the box label.

Further information

- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- 2 ml collection tubes are provided.

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 µl of Solution C1 and invert several times or vortex briefly.
   Note: Solution C1 may be added to the PowerBead tube before adding soil sample
3. Secure PowerBead Tubes horizontally using a Vortex Adapter tube holder (cat. no. 13000–V1–24).
4. Vortex at maximum speed for 10 min.
   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Centrifuge tubes at 10,000 x *g* for 30 s.
6. Transfer the supernatant to a clean 2 ml collection tube.
   Note: Expect between 400–500 µl of supernatant. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extractions with this incubation we recommend you retain the step.
8. Centrifuge the tubes for 1 min at 10,000 x *g*.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
    Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step.
11. Centrifuge the tubes for 1 min at 10,000 x *g*.
12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml collection tube.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow through.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow through. Centrifuge again for 1 min at 10,000 x *g*.
18. Carefully place the MB Spin Column into a clean 2 ml collection tube. Avoid splashing any Solution C5 onto the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-Free PCR Grade Water for this step (cat. no. 17000–10).
20. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.
    Note: Solution C6 is 10 mM Tris-HCl, pH 8.5. We recommend storing DNA frozen (–20° to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

Sample to Insight — QIAGEN

Appendix 3: Table with results from DNA analysis sorted by OTU

OTU	Putative taxon	Total Score	Coverage	Identity	E value	Sample ID
14	*P. cambivora*	1624	97%	99,89%	0	C1, C3, C4, C5, U2, U4, U5, FO1, FO2, FO3, FO4, FO5, FB6, FB7, FB8, FB9, FB12, FB13
69	*P. infestans*	46,4	3%	100,00%	0,0103372	C3, C5, U1, U5, FO1, FO2, FO3, FO4, FO5, FB6, FB7, FB9, FB12, FB13
524	*P. cinnamomi*	44,6	3%	100,00%	0,0361808	U1, U5, FO1, FO4
761	*P. capsici*	45,5	3%	96,30%	0,0344722	U3, FO4, FB13
1167	*P. cinnamomi*	89,1	4%	100,00%	0,0284723	U4, FO3
1392	*P. cambivora*	1574	61%	98,98%	0	U3, FO2
2078	*P. cambivora*	1149	76%	99,06%	0	FO4, FB8
2634	*P. cinnamomi*	91,8	11%	100,00%	0,00085153	FB10
3145	*P. cambivora*	706	57%	98,50%	0	FB8
3545	*P. cambivora*	1240	81%	97,65%	0	FB8
3702	*P. cambivora*	1769	42%	97,66%	0	U3, U5
4396	*P. cambivora*	1626	55%	99,89%	0	C3, C4, C5, U5, FO2, FB9
4664	*P. cambivora*	1157	78%	98,62%	0	FB8
4897	*P. citricola*	1606	57%	99,76%	0	U1
5446	*P. cambivora*	1589	70%	98,45%	0	C4, C5

Appendix 4: Mean values and SD (n=3) of soil microbial community profiling after 96 h of incubation

Sample ID	Time (h)	AWCD	R (Richness)	H'Diversity)	E (Evenness)
FB6	96	0,21 (+-0,04)	20 (+-7,64)	2,95 (+-0,47)	0,98 (+-0,02)
FB12	96	0,13 (+-0,01)	5 (+-2,52)	1,59 (+-0,43)	0,99 (+-0,02)
FO1	96	0,19 (+-0,04)	20 (+-4,16)	2,97 (+-0,28)	0,99 (+- 0,00)
FO2	96	0,48 (+-0,03)	24 (+-2,31)	3,09 (+-0,10)	0,97 (+-0,00)
U1	96	0,38 (+-0,05)	27 (+-3,51)	3,22 (+-0,14)	0,98 (+-0,00)