Masterproef
Diversity and naphthalene degrading potential of epiphytic fungi on hornbeam leaves in a crude oil contaminated area

Promotor :
dr. Sofie THIJS

Anneleen Thoonen
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
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LIST OF ABBREVIATIONS

AAT	Area under absorbance versus time curve
AH	Aromatic hydrocarbon
ANOSIM	Analysis of similarities
ARISA	Automated ribosomal intergenic spacer analysis
FDA	Fluorescein diacetate
ITS	Internal transcribed spacer
MAH	Monocyclic aromatic hydrocarbon
MEA	Malt extract agar
NMDS	Non-metric multidimensional scaling
OUT	Operational taxonomic unit
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
ppmV	Parts per million by volume
PTR-TOF-MS	Proton-transfer-reaction time-of-flight mass spectrometry
VOC	Volatile organic compound

ABSTRACT

INTRODUCTION: Fungi are known to degrade recalcitrant compounds such as monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs) in soils. These compounds are also main constituents of air pollution, mainly due to the burning of fossil fuels. Therefore, it is hypothesized that epiphytic fungi inhabiting the phyllosphere of contaminated areas can degrade these airborne pollutants.

MATERIAL & METHODS: Fungi inhabiting the leaves of hornbeam trees growing in a crude-oil contaminated area in Bóbrka (Poland) and in the polluted city center of Warsaw were isolated and characterized for their potential role in air pollution mitigation. Total DNA from leaves was extracted from the polluted areas and a non-polluted area (Białowieża National Park) to compare epiphytic fungal communities. The isolated fungi were genotypically identified, tested for laccase and peroxidase enzyme production, hydroxyl radical production, tolerance to and degradation of naphthalene and benzene.

RESULTS: Fungal communities inhabiting the phyllosphere of the non-polluted area were significantly different compared to the communities form the polluted city center of Warsaw. A difference in community structure was also seen between the epiphytic communities of Bóbrka and Białowieża, however this difference was less remarkable. Among the epiphytic fungi that were isolated from Bóbrka, *Fusarium sporotrichiodes* AT11, *Phoma* herbarum AT15 and *Lophiostoma* sp. AT37 produced laccase enzymes with activities of 1.24, 3.62 and 7.2 µU.L^{-1} respectively and peroxidase enzymes with activities of 3.46, 2.28 and 7.49 µU.L^{-1} respectively. Additionally, *Fusarium sporotrichiodes* AT11 *and Phoma herbarum* AT15 seemed to tolerate exposure to the contaminants, however, growth was affected after 7 days. *Lophiostoma* sp. AT37 appeared more tolerant to exposure of these contaminants compared to the latter two.

DISCUSSION & CONCLUSION: The results indicated that air pollution plays a role in shaping fungal epiphytic communities. Furthermore, *Lophiostoma* sp. AT37 seemed the best naphthalene degrader of all tested fungi, it had the relatively highest activities of the degradative enzymes and despite the reduced growth after exposure, the fungus seemed still able to metabolize the contaminants.

SAMENVATTING

INTRODUCTIE: Eerder onderzoek heeft aangetoond dat schimmels verbindingen zoals monocyclische aromatische koolwaterstoffen (MAKs) en polycyclische aromatisch koolwaterstoffen (PAKs) kunnen afbreken in bodems. Deze verbindingen zijn ook belangrijke componenten van luchtvervuiling. Ze komen voornamelijk in de atmosfeer terecht door verbranding van fossiele brandstoffen. In deze thesis wordt verondersteld dat epifitische schimmels, die leven in de fyllosfeer (op de bladeren) van bomen, deze luchtvervuilende componenten kunnen afbreken.

MATERIAAL EN METHODEN: Schimmels die leven op bladeren van haagbeuk bomen, welke groeien in een gebied vervuild met ruwe aardolie in de buurt van Bóbrka (Polen) en in de buurt van het vervuilde stads centrum van Warsaw, werden geisoleerd. De mogelijke rol van deze schimmels in het verminderen van luchtvervuiling door aromatische koolwaterstoffen werd bestudeerd. Het totale DNA van blad-wasstalen werd geextraheerd van de twee vervuilde gebieden en een niet-vervuild gebied (Nationaal Park van Białowieża) om de structuur van de schimmel poppultaties tussen de verschillende gebieden te vergelijken. Verder werden schimmels geisoleerd en opgekweekt uit de blad-wasstalen van de vervuilde gebieden en getest voor laccase en perodixase enzymproductie, hydroxylradicaal productie, tolerantie en degradatie van naftaleen en benzeen.

RESULTATEN: Bovenstaand beschreven onderzoek toont een significant verschil aan tussen de schimmel populaties van het niet-vervuilde gebied en het centrum van Warsaw. Er werd ook een verschil gezien tussen de populaties in de fyllosfeer van Białowieża en Bóbrka, hoewel dit verschil kleiner was. De geisoleerde schimmels van Bóbrka: *Fusarium sporotrichiodes*, *Phoma herbarum* en *Lophiostoma* sp. produceerden laccase enzym met activiteiten van 1.24, 3.62 and 7.2 μU.L^{-1} en peroxiase enzymen met activeiten van 3.46, 2.28 and 7.49 μU.L^{-1}. Na blootstelling van deze schimmels aan de contaminanten, werd de groei van de schimmels *Fusarium sporotrichiodes* en *Phoma herbarum* het meest gereduceerd. *Lophiostoma* sp. bleek meer tolerant te zijn na blootstelling, in vergelijking met de andere twee.

DISCUSSIE EN CONCLUSIE:De resultaten geven een indicatie dat luchtvervuiling een rol speelt en het vormen van de schimmel populaties. Bijkomend bleek *Lophiostoma* sp. de beste schimmel voor naftaleen afbraak van de geteste schimmels. Deze had de relatief hoogste enzym acitviteiten, en hoewel groei werd gereduceerd na blootstelling, was de schimmel nog steeds instaat om de contaminant te metaboliseren.

INTRODUCTION

The atmosphere of Earth is the layer of gases, also known as air, that surrounds our planet and is retained by Earth's gravity. The atmosphere protects life on Earth by absorbing ultraviolet (UV) solar radiation, warming the surface through heat retention (greenhouse effect) and reducing temperature extremes between day and night (the diurnal temperature variation) (1). Besides gases (nitrogen, oxygen, carbon dioxide, argon and other trace gases) it also contains variable amounts of dust, smoke and particulate matter, many originating from anthropogenic sources which can have substantial impacts on human life (1,2).

1.1 AIR POLLUTION AND THE CHALLENGE OF REMEDIATION

Since its origins, the atmospheric gases have been subjected to changes in its chemical composition due to both natural (season, weather, time of day, longitude, elevation, geography) as well as anthropogenic processes. The growing world population and the increase of industrial, agricultural and household development has significantly affected air quality leading to a condition of air pollution (3).

Air pollution constituting of gaseous, solids and liquid chemicals in harmful concentrations, has gathered great concern. Especially since the mid-twentieth century when the air pollution disasters of the Meuse Valley fog of December 1930 in Belgium and London's 'Great Smog' in 1952 had taken many lives. Therefore, stringent regulations were established to improve air quality and in particular to limit the adverse effects of air pollution on the public health (4,5). As a result, the European Environment Agency reported an improvement in air quality over the last decades due to reduced emissions. Nevertheless, air pollution concentrations are still too high and European air quality standards and especially World Health Organization (WHO) Air Quality Guidelines (AQGs), which are more stringent, are still being exceeded, particularly in urban areas (6,7).

Air pollution thus occurs when chemicals are present in concentrations that harm or discomfort many organisms. The air pollutants introduced into the atmosphere can occur in a gaseous phase or as particles such as solids and liquid aerosols, from natural or anthropogenic origins. A further distinction can be made between primary air pollutants, emitted directly into the atmosphere from their sources and secondary air pollutants, which are formed due to the reactions between primary pollutants and other elements in the air. Important gaseous air pollutants are ammonia (NH_4), sulfur dioxide (SO_2), nitrogen oxides (NO_x), carbon monoxide (CO), persistent organic pollutants (POPs) and volatile organic compounds (VOCs) (7).

Among VOCs, semi-volatile VOCs such as the monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs) are catching the attention of the international scientific community because of their adverse effects on human health, as well as for their resistance towards degradation and persistence in the environment. MAHs and PAHs can originate from different sources, anthropogenic as well as natural (**Figure 1**). Though, they are mainly formed during thermal decomposition of organic material and their subsequent recombination, under hypoxic (low

oxygen) or anoxic conditions (8). The burning of fossil fuels (*e.g.* diesel fuel) is the main source we are all exposed to on a daily basis (9).

Examples of MAHs include compounds such as benzene (**Table 1**), toluene, ethylbenzene and xylene (BTEX), all these structures have only one aromatic ring (10). Compounds with two or more fused aromatic rings are defined as PAHs. They are comprised of a very diverse group with physicochemical properties that differ for different PAHs (8,11). PAHs commonly found in urban areas include phenanthrene, naphthalene and fluorene (**Table 1**). My research focused on two compounds: benzene (MAH) and naphthalene (PAH). Globally these are two main components measured from the exhaust of cars which use diesel fuels (12).

Table 1. Physicochemical properties of MAHs and PAHs. Molecular formula (M.F.), boiling point (B.Pt), melting point (M.Pt), vapor pressure (V.P.), aqueous solubility (Aq.Sol.) and International Agency for Research on Cancer classification (IARC).

Compound	M.F.	B.Pt. (°C)	M.Pt. (°C)	V.P. (Pa at 25°C)	Aq.Sol. (mg/l)	IARC
Naphthalene	$C_{10}H_8$	218	80.2	11	20	2B
Phenanthrene	$C_{14}H_{10}$	340	100.5	2×10^{-2}	$1 - 2$	3
Fluorene	$C_{13}H_{10}$	295	117	8×10^{-2}	1.69	3
Benzene	C_6H_6	80	5.6	13×10^3	1.79×10^3	1

Aromatic hydrocarbons (AHs) can be present in each compartment of the environment (**Figure 1**). However, the atmosphere receives the relatively largest amount of the AH environmental load (8). When AHs are released in the air they can occur in two phases, a vapor phase and a solid phase. In the solid phase, the AHs can be adsorbed onto particulate matter, depending on their molecular weight and vapor pressures. Naphthalene, phenanthrene, fluorene and benzene have low molecular weights (MW< 200) and are therefore almost exclusively found in the gas-phase (13,14).
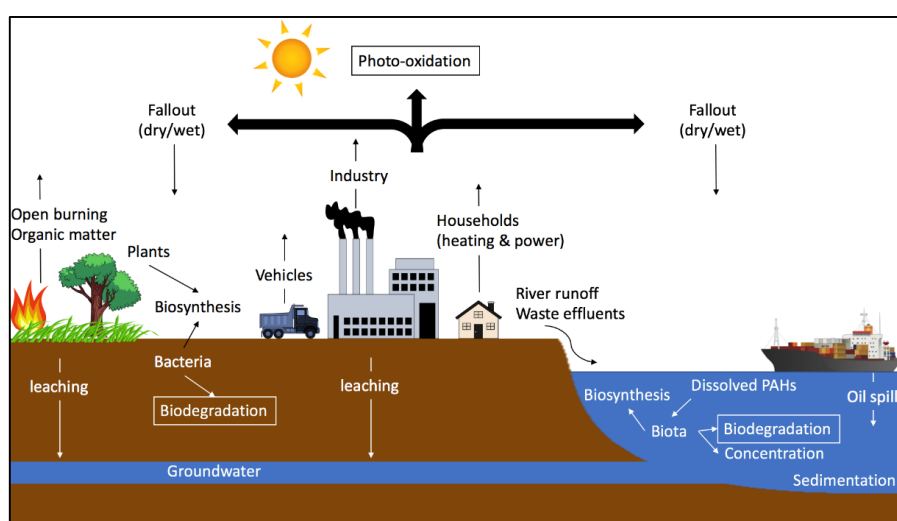

Figure 1. The environmental cycling of MAHs and PAHs, adapted from (15).

In the environment, AHs may be subjected to several processes: adsorption, volatilization, photolysis, chemical degradation, and microbial degradation (16). However, because of their physicochemical properties, low water solubility and highly stable ring structure, they do not degrade easily under natural conditions and have the tendency to persist in the environment (9).

1.1.1 TOXICITY AND HEALTH EFFECTS

Because of their recalcitrant nature and persistence in the environment, exposure to AHs is inevitable. Moreover, the general population is usually exposed to a mixture of these compounds, rather than one type of AHs (17). Many of these compounds can have adverse effects on human health. Studies on laboratory animals have shown that some AHs and especially their reactive metabolites can have mutagenic and/or carcinogenic activities. It is suggested by the National Toxicology Program, Department of Health and Human Services (18), that the mechanisms by which AHs exert their mutagenic activity is similar among the different AHs. Metabolic processes lead to more reactive forms of these compounds eventually forming DNA adducts which can ultimately result in mutations (18,19). Furthermore, evidence from epidemiological studies on occupational exposure indicate an increased risk on skin, lung, bladder and gastrointestinal cancers (20,21).

Since AHs are ubiquitously present in the environment and because of the toxic effects they can exert, strategies for removing these airborne pollutants are necessary. Remedial measures to improve air quality are mainly based on sequestration of air pollutants by classical filters (*e.g.* smoke-filters in industry, cars, buses, tunnels…). In addition, prevention measures and institutional laws are important to reduce the levels of future emissions. (22). Despite this synergic approach, air pollution concentrations are still too high especially in urban areas, therefore, green, sustainable, and cheap methods are urgently required to remove current air pollutants and improve environmental air quality. It is believed that plants and their microbiome can assist in achieving this goal (23).

1.2 THE PHYLLOSPHERE MICROBIOME OF PLANTS

Plants are known to scavenge a substantial amount of fine dust and air pollutants. Especially plant leaves adsorb pollutants passively by physical precipitation and adsorption, uptake or chemical transformation (24). Because of the significant surface area they span on Earth, which is estimated as a global leaf area of 508,630,100 km^2, leaves can have a substantial impact as a method for filtering air (25). Plant leaves are not only a passive surface area for air filtration, it is also the habitat for thousands microbial cells including fungi and bacteria (23).

The phyllosphere can be defined as the aerial parts of living plants, which includes leaves, stems, buds, flowers, and fruits that provide a habitat for microorganisms (26). Studies on phyllosphere microbiology have been mainly focusing on leaves, which are the most dominant plant structures. The microbial communities on leaves are diverse and include many genera of bacteria, fungi, yeasts, algae and, less abundant, protozoa and nematodes. Though, the most dominant colonizers of the phyllosphere are bacteria with 10^6 to 10^7 cells.cm^{-2} of leaf. For fungi, the number of cells are expected to be lower (26). Fungal communities on leaves are strongly dominated by members of

the Ascomycota and the most abundant taxonomic groups from this phylum are the saprotrophs, pathogens and lichenized fungi (27).

Fungi colonizing the surface of leaves are known as epiphytes (**Figure 2**), whereas fungi that reside within the plant tissues are called endophytes. However, the distinction between these two groups is vague, since epiphytes can also penetrate the leaf surface of the plant (28). These Fungi living on leaves can either effect the plant negatively as pathogens (29), or can have a positive effect through protection against pathogenic infections and reducing herbivory by producing toxic metabolites (30). Studies by Jumpponen and Jones (27) also indicate that the development of pathogenic fungi is repressed by saprotrophic phyllosphere fungi.
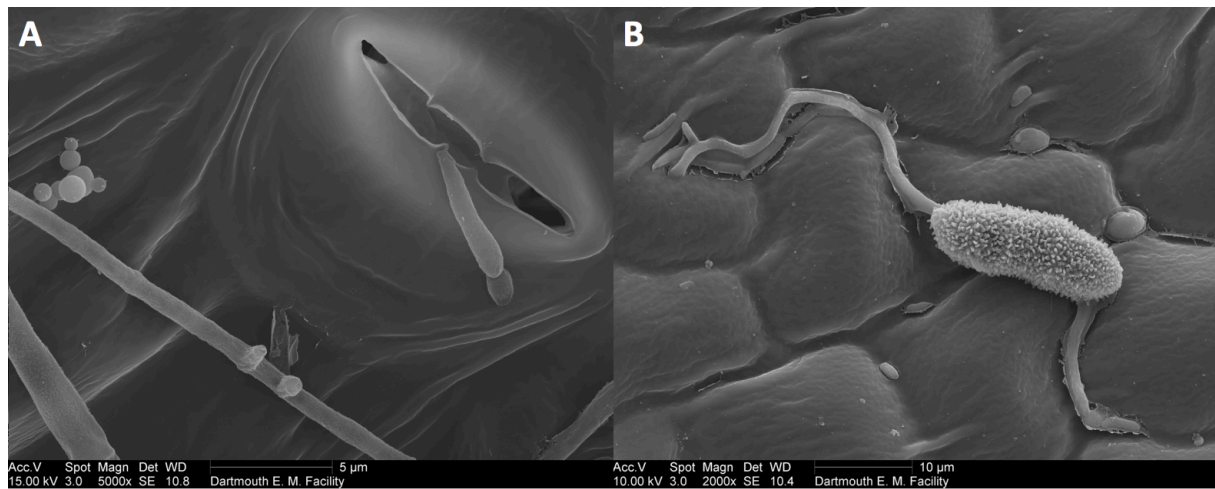
Figure 2. Epiphytic fungi on leaves. Scanning electron microscope (A) image of stomata on lower leaf surface, with fungal hyphae and fungal spores present and (B) fungal hyphae growing from a spore on the lower leaf surface (leaves from *Malus domestica*) (31).

1.3   IMPACT OF AIR POLLUTION ON EPIPHYTIC MICROBIAL COMMUNITIES

Phyllosphere microorganisms, since they are continuously in contact with air and consequently with the airborne AHs, can play an important role in degrading and filtering air pollutants and consequently improving air quality (32). Though, these microbial communities can be affected by several biotic and abiotic factors. They have adapted to a harsh life where nutrients and water are limited. In addition, in this environment there is competition for nutrients and space by other microorganisms and exposure to UV light, large temperature shifts, reactive oxygen species (ROS) and (air) pollutants (25,26).

1.3.1   FUNGAL PHYLLOSPHERE MICROBIOME

Studies by Kembel and Mueller (33) and Jumpponen and Jones (27), present evidence for a great diversity of fungal communities on the surface of leaves in different ecosystems. In tropical regions, a greater phyllospheric fungal diversity is found with an average of 424 fungal OTU richness compared to 50 – 120 fungal OTUs in temperate regions.

However, these phyllospheric fungal communities are dynamic and can be influenced by several environmental factors, such as air pollution (34). For example, Kannangara and Sirisena presented evidence that fungal communities of ornamental plants on roadsides in Sri Lanka adapt to pollution conditions shaping a community that is able to degrade pollutants, and more specifically AHs (35). Other effects of air pollution on a wider diversity of plant and fungal species are largely unknown to date.

1.3.2 BACTERIAL PHYLLOSPHERE MICROBIOME

Bacterial communities are the most dominant colonizers of leaves. However, these communities can also be affected by air pollution and are even more susceptible for air pollutants than fungi according to Brighignal (34). More information concerning the bacterial phyllosphere microbiome has been described by Vorholt (25), Müller (32) and Weyens (23).

1.4 PHYLLOREMEDIATION

Phylloremediation is a green technology, which is based on the synergistic action of plants and their associated microorganisms to degrade airborne pollutants (Error! Reference source not found.). Engineers and plant scientists are focusing on harnessing the potential of plants and their associated microorganisms to mitigate air pollution (36). To date very little is known about the interactions of microorganisms on plant leaves. More specifically, which microbes are present and which mechanisms are used by these microbes to degrade airborne pollutants. This lack of knowledge is a main impediment to further investments and installations of urban green with the purpose for phylloremediation. For example, VOCs capture and degradation is very much dependent on plant type, height, size, biomass, and properties such as leaf surface area, needle-trees or evergreen plants. Other factors determining the capture or air pollutants are percentage porosity in plantings, and also the presence of air pollutant degrading microorganisms, which determine air pollutant degradation efficiency (23,37).
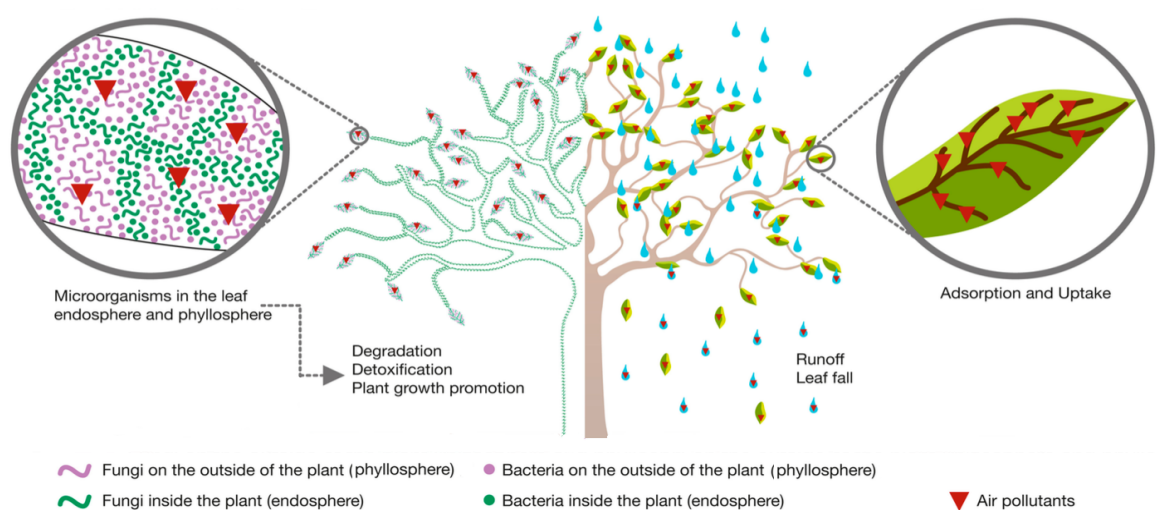

Figure 3. Schematic overview of phylloremediation of air pollution, adapted from (23).

Studying the contributions of the microorganisms to air pollutant mitigation is challenging because of the numerous complex interactions between microbes and with their host. Though advances in culture-independent techniques such as next generation sequencing techniques and advanced analytical methods in combination with improvements in culturing methods have enabled already a better understanding of the phyllosphere microbiology over the last years (32).

1.5 ROLE OF EPIPHYTIC FUNGI IN ORGANIC AIRBORNE POLLUTANT DEGRADATION

Knowledge on how epiphytic fungi degrade AHs has been based for many years on the information we have from plant-associated mycorrhizal fungi in soil and free-living saprotrophic white-rot fungi. We have little understanding about which enzymes are present in epiphytic phyllospheric fungi to degrade AHs, or which other mechanisms are used (for example radical production) (38). Below we discuss therefore the most important enzymes known to break down AHs in basidio- and ascomycetes.

1.5.1 FUNGAL DEGRADATION PATHWAYS OF MAHS AND PAHS

Fungi capable of degrading AHs can be divided into two groups based on the enzymes they produce: ligninolytic and non-ligninolytic fungi. Ligninolytic fungi are able to breakdown AHs by producing extracellular ligninolytic enzymes such as laccases, lignin- and manganese-peroxidases, whereas in non-ligninolytic fungi it is the cytochrome P-450 monooxygenases which plays an important role in the degradation of organic pollutants (39).

In general, the degradation of AHs involves the breakdown of these organic compounds into less complex and therefore, less toxic metabolites through biotransformation, and further degradation into inorganic minerals such as H_2O and CO_2 (aerobic), or CH_4 (anaerobic), through mineralization (9). Fungi can use several different mechanisms to degrade these pollutants indicated in **Figure 4**
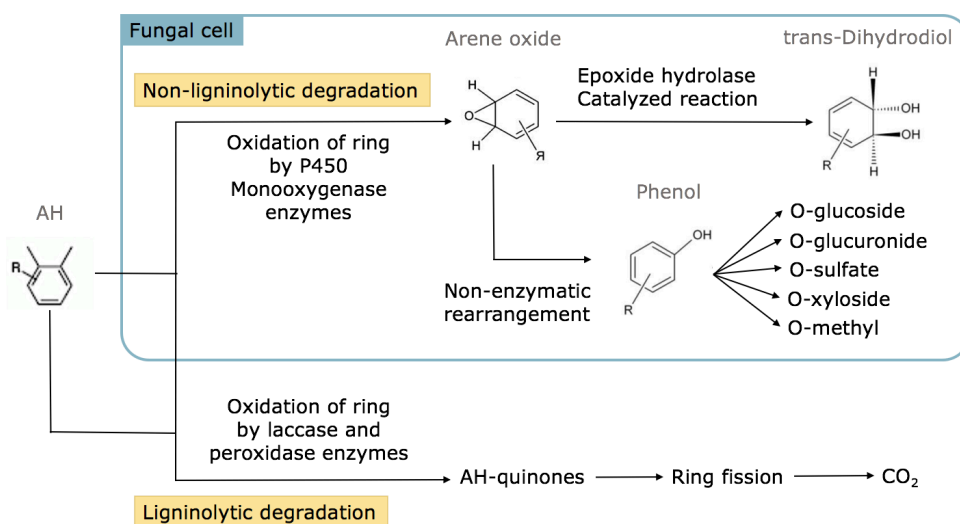

Figure 4. Two main pathways used by fungi to degrade AHs. Adapted from (40).

Ligninolytic fungi, also known as white-rot fungi, are basidiomycetes which are mostly found in soils and less in the phyllosphere. These fungi produce enzymes to degrade lignin, a component of wood or litter. Besides lignin degradation, these enzymes are also capable of degrading compounds, which are structurally similar to lignin such as many organic chemicals including AHs (**Figure 4**). Lignin as well as AHs contain stable aromatic rings in their structures. Due to the irregular structure of lignin, ligninolytic enzymes have a very low substrate specificity which makes them suitable for degrading various organic compounds (41). An example of a white-rot fungus that occurs in the phyllosphere, more specifically on stems of trees, is *Ganoderma* sp.. Torres et al. indicated that the *Ganoderma* sp. UH-M stain was able to degrade PAHs (42).

Non-ligninolytic fungi, including ascomycetes, are also able to degrade various xenobiotic compounds (**Figure 4**). The enzymes used by these fungi are classified as phase I and phase II enzymes. These metabolic enzymes are common in all eukaryotes and catalyze xenobiotic biotransformation and detoxification reactions (43). Phase I reactions are catalyzed by cytochrome P450 monooxygenases and epoxide hydrolases. Important phase II enzymes are for instance glutathione S-transferases (GST), NAD(P)H quinone oxidoreductase and UDP-glucuronosyltransferases (43,44). Besides the phase I and II enzymes, some ascomycetes can also produce laccases and peroxidase enzymes or hydroxyl radicals to induce extracellular oxidation of xenobiotics (45–47).

1.6 SELECTION OF THE HOST PLANT FOR PHYLLOREMEDIATION: HORNBEAM

Plants in urban areas are mainly chosen for aesthetics, low maintenance and not hindering human activities. An ornamental tree that is often used in cities and parks is the European or common hornbeam (**Figure 5A**). The European hornbeam (*Carpinus betulus*) is a tree species native of European countries and Turkey. It is a deciduous tree which is considered as a medium sized tree, reaching heights of 15 to 25 meters. Hornbeam is commonly planted as a street tree, because it is very densely-foliated, and its columnar or oval-shaped growth, this tree is also ideal for the use as a hedge, screen, or windbreak (48,49).

Figure 5. Hornbeam (*Carpinus betulus*) (A) trees in a city center, (B) leaves of the hornbeam tree.

The leaves of the hornbeam tree (**Figure 5B**) are elliptical, heavily textured with very impressed veins, in summer they have a dark green color and in autumn they turn yellow and orange. In winter when leaves turn brown they still remain for a long time attached on the trees (48,49). Because of the heavily ribbed structure, fine dust and air pollutants adhere to the leaf surface.

1.7 INCREASING OUR KNOWLEDGE ON HORNBEAM EPIPHYTIC FUNGI AND THEIR DEGRADATION CAPACITIES

Most of the research on AH degradation by fungi has been done in soils (9), not much is known about degradation of AHs in the phyllosphere. Therefore, it is interesting to investigate if the fungal communities inhabiting the phyllosphere have the capacity to degrade these pollutants.

Phyllospheric fungi inhabiting the leaves of hornbeam trees (*Carpinus betulus*) in Bóbrka (Poland) have been selected for this project. In the forest near Bóbrka, the world's first oil field was established, with a regular oil output since 1854 (50). It might be that fungi growing on these leaves can tolerate and possibly degrade AHs. Chances to find a good degrader in the area of Bóbrka are high because of the 1000 years of crude oil drilling and AH VOCs permeating the air.

Therefore, we believe that epiphytic fungi on leaves of hornbeam trees in a crude oil-contaminated area can degrade airborne AHs. It is not yet known what the impact is of air pollution on the fungal phyllosphere communities in the polluted areas of Bobrka and in other Polish locations like the city center of Warsaw. But it is expected that fungal phyllosphere communities in polluted areas are different from non-polluted areas and that pollution has shifted phyllosphere communities towards fungi that are more able to degrade pollutants. Also, if these fungi area able to degrade the pollutants, it is unclear by which mechanisms they perform the degradation. However, it is expected that the epiphytic fungi use similar mechanisms to degrade AHs as are used by fungi degrading AHs in soils.

1.8 AIM AND OBJECTIVES

The overall aim of this study is to increase our insights in the community structure and function of epiphytic fungi associated with common hornbeam. For this we specified the following objectives namely to, (I) genotypically characterize the fungal communities on leaves of hornbeam, (II) culture some representatives and identify them by sanger sequencing of the ITS and TEF region, (III) test them for laccase and peroxidase enzyme activity, (IV) perform *in vitro* test for air-borne pollutant degradation, and at last (V) test fungal plant pathogenicity. To reach these objectives, advanced technologies were used, such as Illumina Shotgun and Sanger sequencing, Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS).

This thesis provides for the first time insights on the fungi associated with hornbeam leaves and how some fungal species can assist the host in degrading naphthalene and benzene. If degraders are found, they might pave the way for future remediation applications.

MATERIALS AND METHODS

1.9   SITE CHARACTERISTICS AND SAMPLING

To investigate the fungal communities residing on leaves of hornbeam trees and to assess their potential for AH degradation, three sites in Poland were selected for sampling. These sites included Białowieża National Park which is Europe's last temperate primeval forest and is considered as a non-polluted environment (52.7229° N, 23.6556° E) (51), Bóbrka Oil Industry Museum as a specific site where the environment naturally was in contact with crude oil since the last glacial period (52.8167° N, 23.9264° E) (50) and Warsaw city center (52.2297° N, 21.0122° E) with considerable air pollution originating from traffic, industry and other human activities (52).

For each sampling site, five locations were sampled and at each location four trees and five leaves per tree were collected for microbiome analyses (4 x 5 = 20 biological replicates per site). Leaves were collected into sterile falcon tubes with 50 ml phosphate buffer (P-buffer) containing per liter: 11.95 g $NaH_2PO_4.2H_2O$, 16.5 g $Na_2HPO_4.7H_2O$, 100 µl Tween 80. Samples were transported to the laboratory at 4°C until further processing.

1.10 LEAF WASH SUSPENSIONS

In order to isolate the epiphytic fungi, tubes containing the leaves in P-buffer were hand shaken for 10 s, sonicated for 3 min at 160W using a Bransonic® ultrasonic cleaner 2510E-MT (Branson Ultrasonics Corp, Danbury, USA), vortexed for 1 min and lastly, shaken in an orbital shaker for 15 min at 240 rpm. Leaves were removed from the tubes and the P-buffer was centrifuged (15 min, 4000 g) to pellet the microbial cells. Supernatant was discarded and the final pellet was resuspended in the remaining 3 ml of P-buffer for culturing of fungi and DNA isolation.

1.11 TOTAL DNA EXTRACTION

One milliliter of the cell suspensions was transferred into sterile 2 ml tubes. Cells were pelleted by centrifugation (10 min; 10,000 g), supernatant was discarded and cells were lysed by homogenization into 180 µl of lysis buffer (1x Tris-EDTA buffer pH 8, 1.2 % Triton X-100 and 20 mg.ml^{-1} lysozyme in Rnase free water) and incubation at 37°C for 30 min. Hereafter, 25 µl of proteinase K and 200 µl of AW buffer from the Qiagen Blood & Tissue kit (Qiagen, Netherlands) was added followed by an incubation at 56°C for 30 min. Further, the DNA isolation protocol of the Qiagen kit was followed. The DNA purity (260/280 nm and 260/230 nm) was checked with the NanoDrop ND-1000 spectrophotometer (Isogen Life Science, Netherlands), the DNA-concentration was quantified using the QuantiFluor® dsDNA System (Promega, Netherlands) and the integrity was assessed by running an aliquot of the DNA on a 1% agarose gel. At last the high quality DNA was stored at -20°C upon further analyses.

1.12 ARISA FINGERPRINT ANALYSES

Automated ribosomal intergenic spacer analysis (ARISA) was used for comparing phyllospheric fungal diversity and community structures of the crude oil contaminated area of Bóbrka, the conserved nature reserve of Białowieża and the city center of Warsaw. ARISA is a PCR-based method

that relies on the length heterogeneity of the internal transcribed spacer (ITS) regions to fingerprint microbial communities (53).

The fungal intergenic spacer regions ITS1-5.8S-ITS2, *i.e.* the region between the fungal 18SrRNA gene and the 28SrRNA gene, was amplified using the primer pair 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3'). First, for the PCR amplification, a mastermix was made containing: 5 µl 10x hifi PCR buffer (Roche, Basel, Switzerland), 2 µl 50 mM $MgSO_4$, 1 µl dNTP mix, 0.1 µl of each primer (0.1 mM), 38.8 µl RNase free water and 0.2 µl Platinum® Taq high fidelity (Invitrogen, California, USA) for a total of 50 µl reaction mixture. To each reaction, 1 µl of DNA sample (15 – 40 ng $µl^{-1}$) from the leaf washes was added. PCR-conditions consisted of an initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, annealing at 57.5°C for 30 s and elongation at 72°C for 1 min and a final extension step at 72°C for 5 min. Second, the amplified reaction products were loaded onto DNA-1000-chips (Agilent Technologies, USA), prepared according to the manufacturer's recommendations. The resulting DNA fragments, which vary in length from 150 to 1500 bp, were separated by means of an Agilent 2100 Bioanalyzer (Agilent Technologies, USA), based on capillary electrophoresis.

The 2100 Expert Software (Agilent Technologies) was used to digitalize the ARISA fingerprints, resulting in electropherograms in ASCII formats which were processed using the StatFingerprints package in the 2.13.0 version of the R project (The R Foundation for Statistical Computing, Vienna, Austria).

1.13 SHOTGUN DNA METAGENOME SEQUENCING

The DNA samples were quality checked on agarose gel and quantified using the fluorescence method ds QuantiFluor® system (Promega, Netherlands). The Illumina Nextera XT kit was used for library preparation and the 100 bp PE sequencing kit was used. Samples were sequenced on the Illumina Hiseq 4000 to generate between 5 and 10 million PE reads per sample.

Fastq sample files were quality filtered using the Fastq-mcf tool with the following settings, -l 50 -q30 -w 4 -x 10 --max-ns 0. Quality filtered forward and reverse reads were submitted to the Kaiju web server for taxonomic assignments (54). For read classification, Kaiju finds maximum (in-)exact matches on the protein-level using the Burrows–Wheeler transform which reaches a higher sensitivity and similar precision compared with other k-mer-based classifiers. Sequences were also submitted to the MG RAST server for taxonomic and functional annotation (55).

1.14 DILUTION PLATING AND FUNGI ISOLATION

First, leaf wash suspensions were used undiluted or diluted 10-fold and spread onto two types of solid media for fungi (mainly molds) isolation including Malt Extract Agar (MEA) (56) and Czapek dox pH 5 (56). Second, fungi were purified from 869 rich media plates pH 7 (57), 1/10 rich LB plates pH 7 (58) and from minimal medium YMAb284 plates pH 7 (adapted from (59), (60,61)). Third, enrichment cultures were set-up in which 1 ml of leaf wash suspensions of Bóbrka were added to 250 ml Erlenmeyers with 100 ml of Bushnell-Haas medium pH 7 (62) containing 0.1 % (w/v) diesel-related aromatic pollutants as sole C-source including: diesel, BTEX (mix of Benzene, Toluene,

Ethylbenzene, o-Xylene, m-Xylene and p-Xylene), PAH (Naphthalene, Fluorene and Phenanthrene), phenol and n-Hexadecane. Pollutants were spiked into the medium or added in Eppendorf tubes taped into the Erlenmeyer flask for gas phase exposure. Cultures were diluted each three weeks (1:50) and after four transfers, aliquots were spread onto MEA and Czapek dox agar. All plates were incubated at 23°C for two to four weeks.

Fungi picked up from the plates were grown on MEA, Czapek dox agar, Ingestad medium (63) and a specific Guaiacol lignin medium (64). MEA and Czapek dox are rich media, while Ingestad is a minimal medium containing only macro- and micro-nutrients and to which diesel was added up to 0.0025% as the sole carbon source. The Guaiacol lignin medium (64) was used to facilitate the selection of fungi that produce enzymes such as laccases and peroxidases. The guaiacol added to the medium acts as a colorimetric indicator with a red halo around the fungus indicating the action of laccase-like enzymes that are produced.

1.15 DNA EXTRACTION OF FUNGAL ISOLATES AND GENOTYPIC IDENTIFICATION

The fungal isolates were cultivated in liquid MEA medium (pH 5) in 12-well plates for one week prior to DNA extraction. Next, DNA was extracted using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., California, USA) and the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Inc., Norcross, Georgia, USA), according the manufacturer's recommendations. Subsequently, a PCR reaction was performed using the primer pair ITS1F (5'-CTT GGT CAT TTA GAG GTA A-3') and ITS4R (5'- TCC TCC GCT TAT TGA TAT GC - 3') to span the entire ITS1-5.8S-ITS2 region in the genome of the fungi. Additionally, the primer pair TEF1-108F (5'- GAY TTC ATC AAG AAC ATG AT-3') and TEF1-1620R (5'- GAC GTT GAA DCC RAC RTT GTC-3') was used to allow finer-level taxonomic resolution of the clades Fusarium and Penicillium.

To perform the PCR amplification, a mastermix was made for each primer pair containing 5.5 μl FastStart 10x reaction Buffer with $MgCl_2$, 1 μl dNTP mix, 2 μl of each Forward and Reverse Primer (0.1 mM), 0.25 μl FastStart HiFi polymerase (Roche Applied Science, Mannheim, Germany), 38.25 μl RNAse free water and 1 μl of 10^{-1} diluted template DNA (2 – 60 ng μl^{-1}). The PCR conditions included an initial denaturation step of 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for ITS/ 50°C for TEF1 primer, extension of the amplicon at 72°C for 1 min and a final extension of 10 min at 72°C (Biorad T100, Bio-Rad Laboratories N.V., Temse, Belgium). The length of the amplicons was checked by gel electrophoresis before sending them for sequencing to Macrogen (Amsterdam, Netherlands).

1.16 ENZYME ASSAYS

Because it was expected that fungi in the phyllosphere could use similar mechanisms to degrade airborne AHs as fungi in soils, enzyme activities for laccase and peroxidase enzymes were determined.

To test laccase and total peroxidase enzyme activities of the epiphytic fungi of Bóbrka and Warsaw, the fungi were first grown in liquid Kimura medium (pH 5). One plug of the fungi was added in a 100 ml Erlenmeyer containing 20 ml Kimura medium and incubated at 23°C for two weeks static.

After this period of growth, the enzyme activities were measured each 48 hours using a FLUOstar®
Omega Plate reader (BMG LABTECH Inc., Ortenberg, Germany). The fungi *Clitocybe dealbata*,
Clitocybe nebularis and *Ganoderma* sp. (42) which are all basidiomycetes and known to produce
laccase and peroxidase enzymes were included as positive controls. The laccase enzyme activity was
determined spectrophotometrically by monitoring the oxidation of 1 mmol.L^{-1} 2,6-dimethoxyphenol
(DMP) to 2,2',6,6'-dimethoxydiphenoquinone in 100 mM sodium acetate buffer, pH 5. For a final
reaction sample of 200 µl, 10 µl of the culture supernatant was added to 186 µl of the sodium acetate
buffer and lastly 4 µl of a 50 mM DMP was added, hereafter the activity was directly measured during
1 min, using a FLUOstar® Omega Plate reader set to a wavelength of 468 nm. Laccase enzyme
activities were expressed in µU.L^{-1} (ε468 nm: 49 600 M^{-1}.L.cm^{-1}) (65).

The total peroxidase enzyme activity was determined using 0.5 mM o-dianisidine as a substrate for
oxidation in the presence of 4 mM H$_2$O$_2$. The activity was determined in a final reaction sample of
200 µl containing 100 µl acetate buffer (100 mM, pH 5), 39 µl H$_2$O, 26 µl of 4 mM H$_2$O$_2$, 10 µl of the
culture supernatant and lastly added, 25 µl of a 4 mM o-dianisidine stock (dissolved in absolute
ethanol). After adding the o-dianisidine, absorbance was directly measured for 1 min at 445 nm.
The peroxidase enzyme activities were expressed in micro units per liter medium (µU.L^{-1}) (ε445 nm:
47 665 M^{-1}.L.cm^{-1}) (66).

1.17 FUNGAL HYDROXYL RADICAL PRODUCTION ASSAY

Fungal hydroxyl radical production was determined by measuring the oxidation of the substrate
terephthalate to hydroxylterephthalate (67). Fungi isolated from the phyllosphere of Bóbrka and
Warsaw and the three positive controls were cultivated in 100 ml Erlenmeyers with 20 ml liquid MEA
medium, in triplicate, for one week at 23°C. After one week, the mycelia were washed with Ingestad,
the old medium was pipetted out and the Ingestad medium was added and left for one hour. After
one hour, the media was taken out and 5 ml of Ingestad with glucose (0.52 g.L^{-1}) and 2.5 mM
terephthalate was added to the Erlenmeyers. Form each Erlenmeyer 100 µl of the culture
supernatant was pipetted in a 96-well plate to measure the absorbance using a FLUOstar® Omega
Plate reader at 311 nm. Measurements were done after one hour and then each day for one week.
Blanks were subtracted from the measurement data and results were expressed as area under the
absorbance versus time curve (AAT).

1.18 MAH & PAH TOLERANCE TESTS

The four most promising epiphytic fungi selected after conducting the enzyme tests and the two
fungi that were considered as positive controls (*C. dealbata* and *C. nebularis*) were used to conduct
an AH exposure and tolerance experiment. To assess the tolerance of the fungi to the AHs, fungal
growth was measured regularly after exposure to naphthalene and benzene. First, fungi were grown
on plates with Ingestad medium containing 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose as carbon source.
When the fungi reached a diameter of 2 cm they were exposed to gaseous naphthalene (10 ppmV)
and benzene (9000 ppmV). For each fungus three replicates were used for the control group and
three for the exposed group. The diameter of the fungi on the plates was measured the day of
exposure and then each two days for up to 11 days. Calculations in Excel were used to determine
the pollutant effect on the growth rate of the fungi.

1.19 NAPHTHALENE AND BENZENE AIRBORNE POLLUTANT DEGRADATION EXPERIMENTS

To assess if the four fungi selected in the previous experiments could degrade aromatic pollutants, a static experiment in closed 500-ml Erlenmeyers with solid media was performed. First, the selected fungi were grown on solid media in two batches, one with Kimura medium (pH 5) and one with Ingestad medium containing 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose (pH 5) in Erlenmeyers with two necks and closed with mininert stoppers with sampling valves. Each batch was again divided based on the growth rate of the fungi in slow-growing and fast-growing. After two weeks of cultivation, when the fungi reached a diameter of approximately 5 to 8 cm, the fast-growing fungi were exposed to naphthalene (10 ppmV) and benzene (9000 ppmV) in the air-phase. After reaching the desired mycelium size (5 – 8 cm) the slow-growing fungi were also exposed to these contaminants.

For each batch of Erlenmeyers, four only medium controls were included. Additionally, controls for the a-specific absorption of the contaminants were included to determine the amount of contaminant that was absorbed in the mycelium of the fungi. This was done by spraying a CuSO$_4$ solution (300 mM) on the fungi to make them metabolically inactive.

After two weeks of exposure to the contaminants, the naphthalene and benzene concentrations and possible degradation products in the air-phase were measured using Proton-Transfer-Reaction Time-Of-Flight mass spectrometry (PTR-TOF-MS).

1.20 PTR-TOF-MS DATA ANALYSES

VOCs were detected in real-time through proton transfer reactions occurring between the H$_3$O$^+$ ions produced within the ion source and the sample gas inserted into the drift tube (68). The drift tube is kept under controlled conditions of pressure (2.3 mbar), temperature (50 °C) and voltage (600 V) resulting in a field density ratio (E/N) of ≈130 Td (E being the electric field strength and N the gas number density; 1 Td = $10-17$ V cm^2). After a performed proton transfer reaction, protonated ions are extracted from the drift tube and pulsed every 30 µs to the orthogonal time-of-flight region to be separated according to their m/z ratio in the time-of-flight before being detected in conjunction with a multi-channel-plate (MCP) and a time-to-digital converter (TDC) (Burle Industries Inc., Lancaster, PA, USA).

Raw 10 Hz time series of high resolved full mass spectra ranging between 1 and 315 m/z were continuously acquired by the TofDaq software (Tofwerk AG, Switzerland) and stored in hdf5 file format in 6-min time periods (69). After acquisition, each file was post-processed by the routine programs of a software designated for PTR-TOF-MS data analysis (68). This allowed an accurate mass scale calibration and peak detection through peak shape analysis, iterative residual peak analysis to detect multiple isobaric peaks per unit m/z, and quantification of the fitted peak areas corresponding to ion signal intensities based on the 6-min sum spectra. In addition, this software performs post-analysis quality assurance checks of the whole outcome produced.

1.21 PTR-TOF-MS DETERMINATION OF VOC CONCENTRATIONS

Theoretical absolute concentrations of VOCs were determined from PTR-TOF-MS measurements via the first order kinetic reaction (70) after applying the correction for sample humidity (71) and for the effect of the duty cycle as proposed by Cappellin *et al*. (72).

$$[VOC] = (1/k\tau)*\left([VOC^{\cdot}H^+]_{measured}/[H_3O^+]_{measured}\right)*$$
$$\left(\sqrt{(m/z)}_{H_3O^+}\Big/\sqrt{(m/z)}_{VOC^{\cdot}H^+}\right)$$

The concentrations $[VOC^{\cdot}H^+]$ and $[H_3O^+]$ are signal ion rates corresponding to the protonated VOC-ions and the primary ion, respectively; τ is the reaction time of $[H_3O^+]$ in the drift tube; k is the reaction rate coefficient between VOC and H_3O^+ according to the tabulation provided by Cappellin et al. (72). When a k for a specific VOC was not available, a standard value of $2*10^{-9}$ cm^3 s^{-1} was used (72). The $[H_3O_{16}^+]$ concentration at m/z = 19.018 was calculated from the natural isotope $[H_3O_{18}^+]$ measured at m/z = 21.022.

The background signal of the PTR-TOF-MS was quantified via an automated system of switching valves that introduced VOC-free air, generated by a commercially available gas calibration unit (Ionicon, Innsbruck, Austria) for 6 min every 6 h and the resulting average value was subtracted from all the previously recorded data.

1.22 GC-MS DATA ANALYSIS

VOCs were also measured using Gas Chromatography Mass Spectrometry (GC-MS). For this we used a 30 meter capillary column DB5-MS, 0.25 mm internal diameter with a film thickness of 0.25 μm (Agilent Technologies). The GC MS parameters used were: 1 μl injection by the TriPlus RSH-autosampler (Thermo Scientific), 30 s splitless mode at 280 °C and split flow at 50 ml min^{-1}. The column temperature was initially 35°C for 1 min, then gradually increased to 245°C at 15°C.min^{-1}. The MS conditions were a scan at 33-100 in 0.2 seconds for benzene and 33-150 in 0.3 seconds for naphthalene. The GC used was a Trace 1310 gas chromatograph (Thermo Scientific) and the MS was a ISQ LT Single Quadrupole Mass Spectrometer (Thermo Scientific). The mass spectra of individual total ion peaks were identified by comparison with the NIST mass spectra database.

1.23 FDA ASSAY

To examine the metabolic activity of the fungi after the AH degradation experiment, a fluorescein diacetate (FDA) hydrolysis analysis was conducted. Two plugs of fungi were added to 10 ml sodium phosphate (60 mM) buffer, 100 μl of FDA solution (2 mg.ml^{-1}) was added and the mixture was incubated while shaking for one hour at 37°C (73). In order to stop the reaction, 400 μl of acetone was added. The mixtures were filtered and the absorbance of the filtrate was measured at 490 nm using a FLUOstar® Omega Plate reader.

1.24 Fungal pathogenicity *in vivo* plant test

To verify that the selected fungi did not incur pathogenic effects on plants (as they were isolated from healthy plants), common Ivy (*Hedera helix*) was inoculated with the fungi. For each of the four fungi, one ivy plant was used and six leaves per plant were inoculated with the fungi by gently pressing the leaves against the mycelia of the fungi on solid medium. After two weeks, plant health was assessed visually by scoring leaf morphology, color and turgor.

1.25 Statistical analysis

All statistical analyses were performed using the 3.2.3 version of R (The R Foundation for Statistical Computing, Vienna, Austria) and the 2.13.0 version of R was used to edit ARISA fingerprints.

For univariate datasets such as the enzyme tests, the tolerance test and the FDA assay, ANOVA and Student's t test were used if the data were normally distributed and homoscedastic. Post hoc analyses for two by two comparisons were performed using Tukey's honest significant differences tests. If the data was not normally distributed, log transformation of the data was done and normality was again checked using the Shapiro-Wilk test. Homoscedasticity of variances was checked using Bartlett's test. If the data was still not normally distributed the nonparametric Kruskal-Wallis test was performed followed by a post hoc analysis using the Pairwise Wilcoxon Rank Sum Test.

Multivariate statistical techniques were applied on the ARISA fingerprints. These were analyzed using non-metric multidimensional scaling (NMDS) with the Bray-Curtis distance metric. Analyses of similarity (ANOSIM) was performed to evaluate significant differences between groups with multivariate data.

1.26 EPIPHYTIC FUNGAL COMMUNITY STRUCTURES

Automated Ribosomal Intergenic Spacer Analyses was carried out to compare fungal communities inhabiting the phyllosphere of hornbeam leaves sampled in different polluted areas in Poland. Precisely, leaves were sampled from the crude-oil contaminated area of Bóbrka, the city center of Warsaw and the non-polluted National Park of Białowieża. NMDS-analysis with the Bray-Curtis distance metric was used to analyze the fingerprints obtained from ARISA analyses (**Figure 6**), with a probability ellipse (standard deviation, p = 0.68) for each different site.

A significant difference was observed between the fungal communities inhabiting the phyllosphere of the hornbeam trees growing in the non-polluted Białowieża National Park and in the polluted city center of Warsaw (ANOSIM; R = 0.8, p = 0.001). Furthermore, the phyllospheric fungal communities of Białowieża and Bóbrka showed differences of their fungal communities (ANOSIM; R = 0.45, p = 0.001). Although, the differences between these communities were less remarkable. A bigger inner variation was noticed in the fungal communities of the phyllosphere of Bóbrka in comparison to the other sampling areas. Fungal communities on each subsite within the Białowieża National Park appeared to differ less than for the other sites. Hence, in Białowieża the smallest between biological sample variation was seen. In addition, differences were visible for the phyllospheric fungi of Bóbrka and Warsaw, however the separation between these communities was less strong (ANOSIM; R = 0.33, p = 0.001).
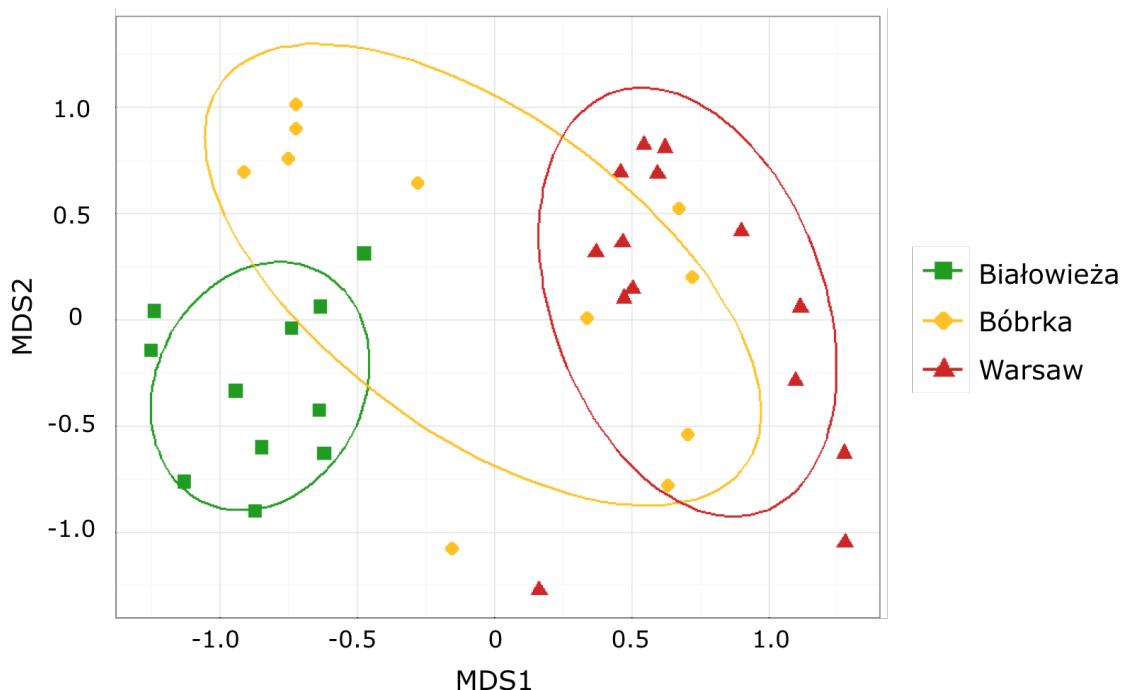

Figure 6. NMDS-analysis of the fungal ARISA fingerprints with the Bray-Curtis distance metric (stress = 0.17) of the Białowieża National Park, the crude-oil contaminated site of Bóbrka and the city center of Warsaw. Probability ellipses (standard deviation, p = 0.68) are shown for each site**.**

1.27 Total and cultivable phyllospheric fungal communities associated with hornbeam

Illumina Shotgun Sequencing was performed to investigate the total epiphytic community composition of leaves sampled from the different sites. Metagenome data obtained from shotgun sequencing was processed using Kaiju, a fast and sensitive metagenome taxonomic classification program. For each site, more than half of the reads were classified (**Table 2**). From these classified reads, the highest relative abundance of fungi could be found in the phyllosphere of Bóbrka (3.09%), followed by Warsaw (1.41%) and last, Białowieża (0.81%). The annotated phyllosphere of each site was clearly dominated by bacteria.

Table 2. Metagenome classification information for each of the three sampling sites.

Site	Classified reads (%)	Fungi (%)	Bacteria (%)
Białowieża	69.01	0.81	98.43
Bóbrka	58.40	3.09	95.87
Warsaw	59.44	1.41	97.57

Relative abundances of the fungal genera for each sampling site are presented in **Figure 7A**. A cut-off value of 1.0% was set, all genera having a relative abundance greater than or equal to 1.0% are presented. The classified fungal genera, with a relative abundance ≥0.1%, belonged to the classes: Dothideomycetes, Sordariomycetes, Leotiomycetes, Taphrinomycetes and Tremellomycetes. All these fungal classes belong to the phylum Ascomycota except for the latter one which belongs to the phylum Basidiomycota. Relative abundances (≥0.1%) of the fungi belonging to the class Tremellomycetes (*Kwoniella*, *Tremella* and *Xanthophyllomyces*), were only detected in the phyllosphere of Bóbrka.

Overall epiphytic fungal communities seemed more diverse in Białowieża and Bóbrka compared to phyllosphere communities of Warsaw. The phyllosphere of Warsaw was clearly dominated by members of the genus *Aureobasidium*, with a relative abundance of 44.17%. Fungi belonging to the genus *Aureobasidium* were also present, although to a lesser extent, in the phyllosphere of Białowieża (8.72%) and in Bóbrka (1.1%). Additionally, the genus *Taphrina*, also detected on all sites, had the highest relative abundance in Bóbrka (6.68%). Though, phyllospheric communities are more diverse in Białowieża and Bóbrka, the community composition and relative abundances of fungal genera also differ greatly when comparing the two sites.

The relative abundances of the phyla Ascomycota and Basidiomycota (**Figure 7B**) present on the leaves of the hornbeam trees on each site differed greatly. Warsaw had the greatest relative abundance of Ascomycota (88%), followed by Białowieża (73%) and last, Bóbrka had the lowest relative abundance (57%). Bóbrka had the highest relative abundance of epiphytic basidiomycetes (43%) compared to the other sites. Remarkably, no basidiomycetes with a relative abundance ≥0.1% could be detected in the phyllosphere of Białowieża and Warsaw. Even though the relative abundance of basidiomycetes in the phyllosphere of Bóbrka is high, the phyllospheric fungal communities on each site are still dominated by fungi belonging to the phylum Ascomycota.
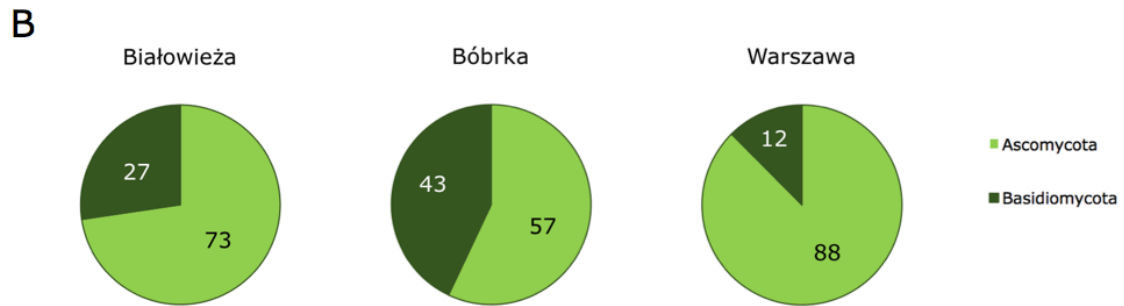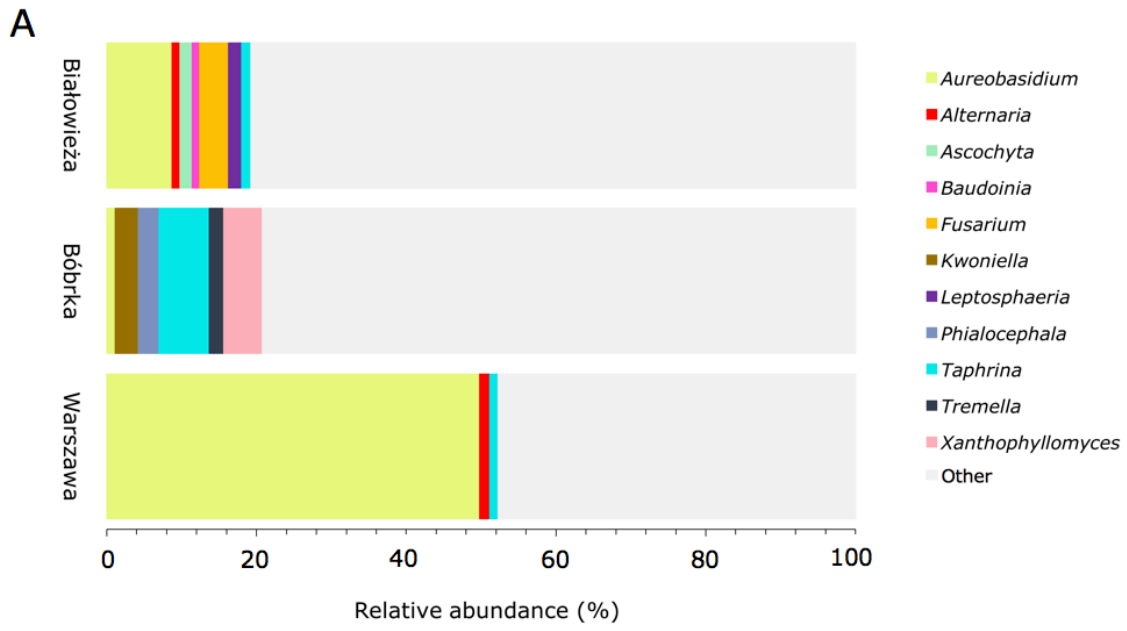
Figure 7. Total epiphytic fungal communities. (A) Relative abundances of classified fungal genera, with a cut-off value of ≥ 1.0%, (B) relative abundances for Ascomycota and Basidiomycota in the phyllosphere, for each site: the non-polluted National Park of Białowieża and the two polluted areas Bóbrka and Warsaw.

The cultivable phyllospheric fungi isolated from the phyllosphere of the crude oil-contaminated area of Bóbrka and the city center of Warsaw were all classified as Ascomycota. These belonged to the classes: Dothideomycetes, Eurotiomycetes, Sordariomycetes (**Table 3**). Fungi belonging to the genera *Alternaria*, *Aureobasidium* and *Fusarium*, detected in the phyllosphere after metagenome analysis, could also be cultivated. The genera *Alternaria* and *Fusarium* were detected in the metagenome of the phyllosphere of Bóbrka. Though, their relative abundances were lower than 1.0%. The fungus *Aureobasidium pullulans*, detected with the highest relative abundance (44.17%) in Warsaw, could only be cultivated form leaf wash samples of Warsaw.

Table 3. Overview cultivated epiphytic fungi from Bóbrka and Warsaw.

Site	Class	Genus	Species	Strains
Bóbrka	Dothideomycetes	*Alternaria*	*alternata*	AT7; AT13; AT14; AT28
	Dothideomycetes	*Cladosporium*	sp*.*	AT23; AT33
	Sordariomycetes	*Fusarium*	*lateritium*	AT6
	Sordariomycetes	*Fusarium*	*sporotrichiodes*	AT8; AT11; AT35
	Sordariomycetes	*Fusarium*	*avenaceum*	AT5; AT17; AT18; AT21
	Sordariomycetes	*Fusarium*	*proliferatum*	AT22
	Sordariomycetes	*Fusarium*	*graminearum*	AT30; AT31; AT36
	Dothideomycetes	*Lophiostoma*	sp*.*	AT37
	Eurotiomycetes	*Penicillium*	*citrinum*	AT12; AT14; AT26; AT27; AT32
	Eurotiomycetes	*Penicillium*	sp.	AT10
	Dothideomycetes	*Phoma*	*herbarum*	AT15; AT16
	Sordariomycetes	*Sarocladium*	*strictum*	AT4
Warsaw	Sordariomycetes	*Acremonium*	*frucatum*	AT3
	Dothideomycetes	*Aureobasidium*	*pullulans*	AT2; AT20; AT25
	Dothideomycetes	*Cladosporium*	sp*.*	AT1

1.28 Enzyme assays

The cultured epiphytic fungi were investigated for laccase and peroxidase enzyme production since it was speculated that the epiphytic fungi might use these enzymes to degrade AHs. Three fungi were used as positive controls: *Clitocybe dealbata*, *Clitocybe nebularis* ST1 and *Ganoderma* sp. UH-M, these are all basidiomycetes producing ligninolytic enzymes including laccase and peroxidase (42).
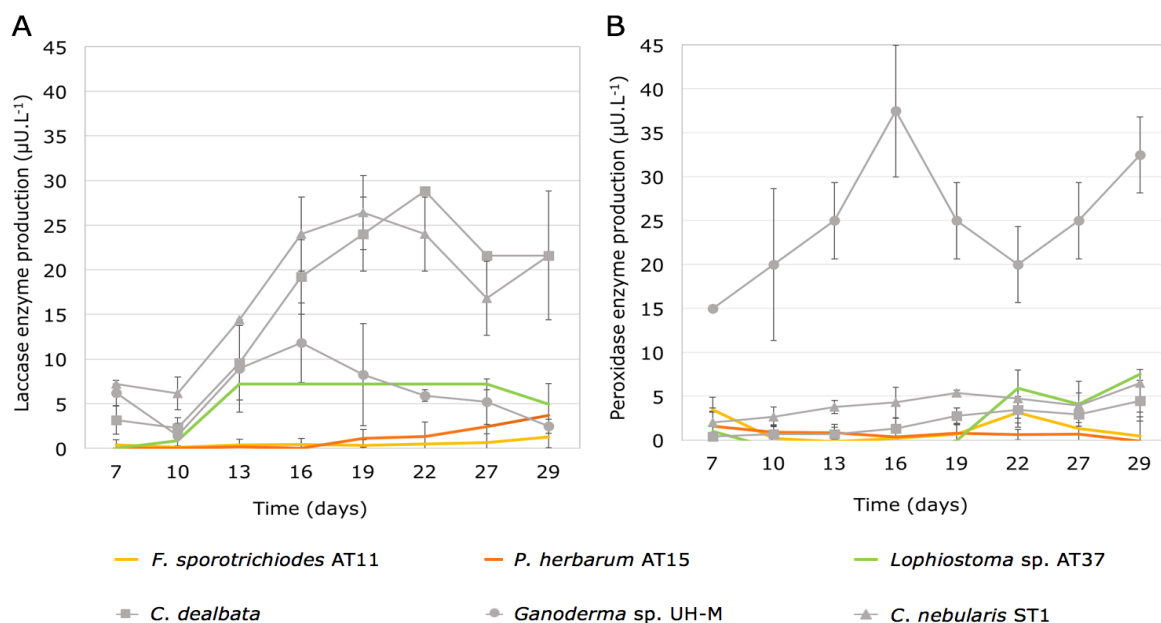

Figure 8. Time course analysis of (A) laccase and (B) total peroxidase enzyme production by epiphytic fungi in Kimura medium. Epiphytic fungi are indicated with different colored lines, positive controls (*C. dealbata*, *C. nebularis* ST1, *Ganoderma* sp. UH-M) are indicated in gray. The means ± SD of the enzyme activities for each fungus are shown.

Among all the selected epiphytic fungal strains, *F. sporotrichiodes* AT11, *P. herbarum* AT15 and *Lophiostoma* sp. AT37 were able to produce laccase and peroxidase enzymes in Kimura medium. The activities of both enzymes were determined starting from the seventh day of incubation. *Lophiostoma* sp. AT37 had the relatively highest enzyme activity for laccase and began producing the enzyme after 10 days, with an enzyme activity of 7.2 µU.L^{-1} at its peak. For 14 days, the enzyme activity of laccase was stable and after this period it declined. The fungi *P. herbarum* AT15 and *F. sporotrichiodes* AT11 began producing the laccase enzymes after 19 and 27 days respectively, with enzyme activities of 3.62 µU.L^{-1} and 1.24 µU.L^{-1} (**Figure 8A**). No statistical significant differences were observed comparing laccase enzyme production for each fungus. However, the Ascomycete *Lophiostoma* sp. AT37 together with the basidiomycetes used as positive controls had the relatively highest laccase enzyme activities.

In general, the enzyme activities for the total peroxidase enzymes and laccase enzymes produced by the epiphytic fungi were comparable. Though, the fungus *Lophiostoma* sp. AT37 began producing peroxidase enzymes later (19 days) compared to the production of laccase enzymes (10 days). In

addition, the peroxidase enzyme activity was slightly higher (7.49 µU.L^{-1}). *Fusarium sporotrichiodes* AT11 already produced these enzymes after 7 days (3.46 µU.L-1). However, after 10 days no enzyme activity was detected anymore, enzyme production started again after 19 days. Peroxidase enzyme activities for the epiphytic fungi P. herbarum AT15 were higher with a peak activity of 2.28 µU.L-1, compared to the laccase enzyme activity. For the peroxidase activity as well as for the laccase activity, the epiphytic fungus Lophiostoma sp. AT37 had the relatively highest enzyme activities compared to the other epiphytic fungi. Concerning the positive controls of the experiment, Ganoderma sp. UH-M seemed to have the highest peroxidase enzyme activity.

1.29 HYDROXYL RADICAL PRODUCTION

Besides laccase and peroxidase enzyme activities, hydroxyl radical production was determined for the epiphytic fungi cultured from the phyllosphere of Bóbrka and Warsaw. The three fungi used earlier as positive controls (*C. dealbata*, *C. nebularis* ST1 and *Ganoderma* sp. UH-M) were also investigated. The hydroxyl radical production for *F. sporotrichiodes* AT11 and *Lophiostoma* sp. AT37 was significantly higher than for *P. citrinum* AT26 and *P. herbarum* AT15. *Ganoderma* sp. UH-M had the significantly highest hydroxyl radical production, hydroxyl radical production for *C. dealbata* and *C. nebularis* ST1 were lower compared to the other strains.
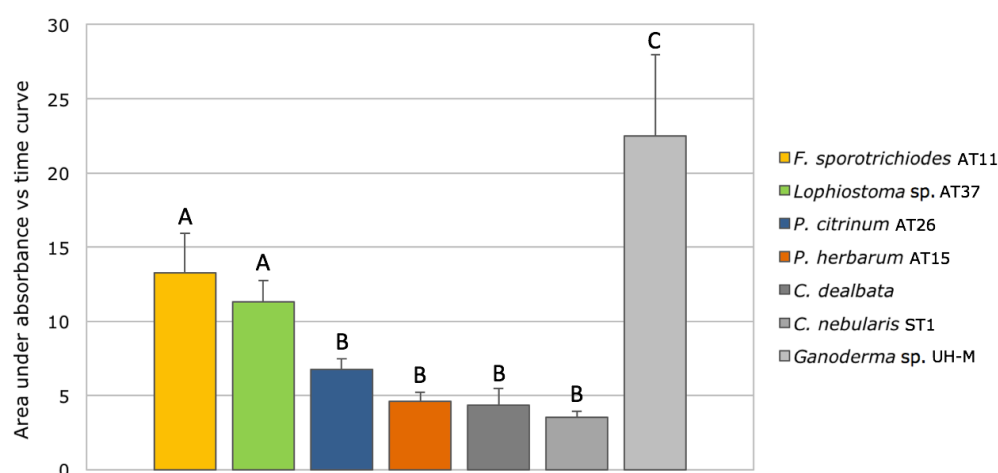
Figure 9. Fungal hydroxyl radical production presented as the area under the absorbance (311 nm) versus time curve. Production of hydroxyl radicals by the epiphytic fungi isolated from Bóbrka and Warsaw together with the three fungi used earlier as positive controls (*C. dealbata*, *C. nebularis* ST1 and *Ganoderma* sp. UH-M) was measured for five days. Statistically significant differences between hydroxyl radical production by the different fungi are indicated with letters above each column, columns with a different letter are significantly different ($p < 0.05$).

1.30 MAH & PAH Tolerance test

To assess the tolerance of the epiphytic fungi to naphthalene (10 ppm) and benzene (9000 ppm) an exposure experiment was performed. The most promising epiphytic fungi, selected in previous experiments for their possible ability to degrade MAHs and PAHs, were exposed to naphthalene and benzene. The net growth rate for each fungus was determined (**Figure 10A**). Growth assessment was ended when the fungal mycelia reached the Petri dish edge. For *F. sporotrichiodes* AT11 and *P. herbarum* AT15 measurements ended after seven days. Net growth rate of these two fungi was significantly lower when exposing them to the contaminants compared to the control conditions (P ≤ 0.01). After two days of exposure, net growth rate still increased but a decline in growth rate was seen after five days for both fungi. The net growth after seven days for *F. sporotrichiodes* AT11 and *P. herbarum* AT15 is indicated in **Figure 10B**. After this period, the net growth was significantly more reduced for *F. sporotrichiodes* AT11 (P. ≤ 0.001) then for *P. herbarum* AT15 (P ≤ 0.01) after exposure. The fungi *Lophiostoma* sp. AT37 and *P. citrinum* AT26 had a lower growth in general. Though, net growth, after 11 days (**Figure 10B**), was significantly lower for *Lophiostoma* sp. AT37 when exposed to naphthalene and benzene compared to the control condition (P ≤ 0.01). Nevertheless, it seemed that the growth rate for this fungus (**Figure 10A**) was not as much reduced (P ≤ 0.05) as for *F. sporotrichiodes* AT11 and *P. herbarum* AT15. When comparing control and exposed conditions for *P. citrinum*, it appeared that not only the growth for the exposed condition was very low, but also for the control condition.
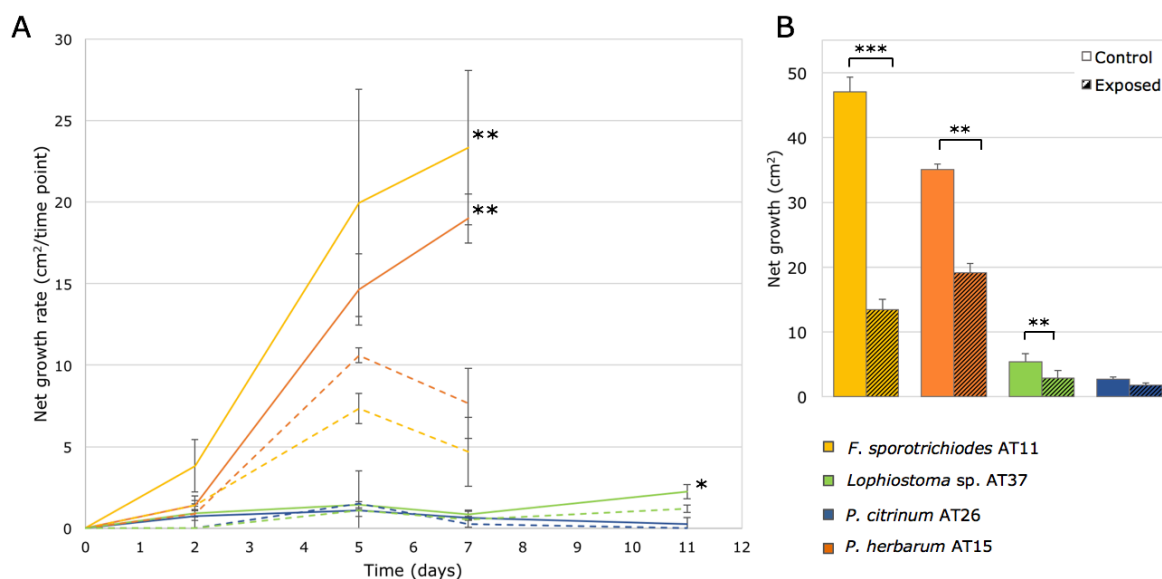
Figure 10. Tolerance test of epiphytic fungi, exposed to naphthalene and benzene, over time. The four selected epiphytic fungi are represented (A) by the colored lines, mean of the net growth rate per time point ± SD for three replicates are presented. A solid line indicates the control condition and a dotted line indicates the exposed condition for each fungus. (B) Net growth, at the last day of the growth assessment, for each fungus. Statistically significant differences are indicated after performing Student's t-tests by: * (P ≤ 0.05), ** (P ≤ 0.01) and *** (P. ≤ 0.001).

Tolerance to naphthalene and benzene of the fungi *C. dealbata* and *C. nebularis* ST1, previously used as positive controls for the enzyme tests, was also assessed. When comparing control and exposed conditions for *C. nebularis* (**Figure 11B**), it appeared that the net fungal growth, after 11 days, was significantly affected after exposure to the contaminants ($P \leq 0.05$). After five days of exposure, net growth rate (**Figure 11A**) declined and at day seven, net growth was significantly lower after exposure ($P \leq 0.01$). After day seven, net growth rate of the exposed fungus, was even more declining. In contrast, the growth of the fungus *C. dealbata* appeared to be less affected by exposure to naphthalene and benzene compared to the control condition (**Figure 11A** and **B**).
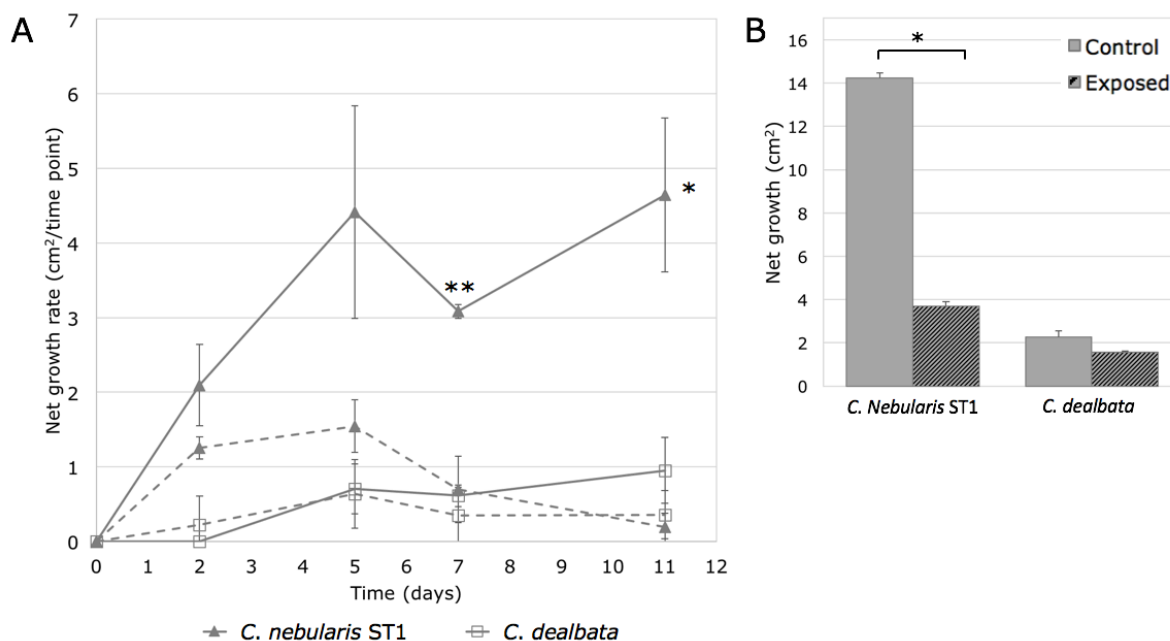
Figure 11. Tolerance test of *C. nebularis* ST1 and *C. dealbata* exposed to naphthalene and benzene. (A) Control conditions are indicated by solid lines and exposed conditions by dotted lines, mean of the net growth rate ± SD for three replicates are presented. (B) Net growth, at the last day of the growth assessment, for each fungus. Statistically significant differences are indicated after performing Student's t-tests by: * ($P \leq 0.05$) and ** ($P \leq 0.01$).

Remarkable was that the pigment production of the fungi *F. sporotrichiodes* AT11 and *P. herbarum* AT15 was altered after exposure to naphthalene and benzene (**Figure 12**). *Fusarium sporotrichiodes* AT11 produced dark pink to red pigments in the control condition on the Ingestad medium containing 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose. However, when exposed to the contaminants the production of the red pigments stopped. Also, a decreased pigmentation was observed for the fungus *P. herbarum* AT15.

Figure 12. Epiphytic fungi exposed to naphthalene and benzene in the tolerance test. Fungi were grown on Ingestad medium with 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose, (A) the fungi *F. sporotrichiodes* AT11 and (B) *P. herbarum* AT15 for both the exposed (top) and control (bottom) conditions are shown.
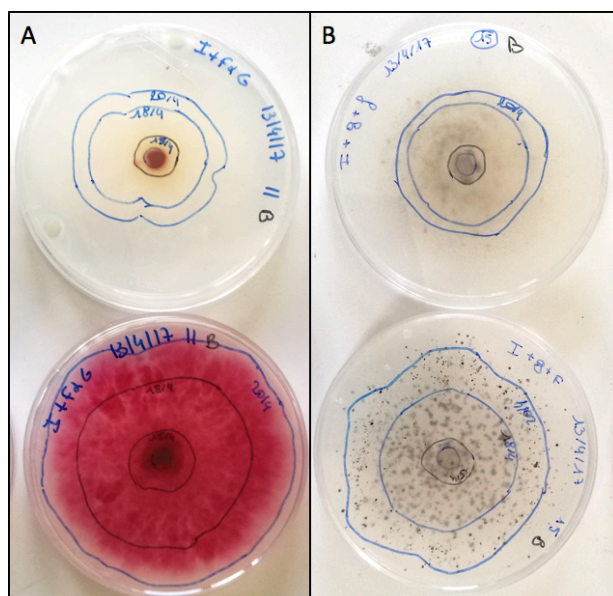
1.31 PTR-TOF EXPERIMENTS

The isolated epiphytic fungi which produced the laccase and peroxidase enzymes and/or the hydroxyl radicals, were selected to assess their naphthalene and benzene degradation potential. Four replicates per fungi were grown static in closed 500 ml Erlenmeyers with solid medium in two batches: one with Kimura medium and one with Ingestad medium containing 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose, including four only medium controls per batch. To determine the a-specific absorption of the contaminants in the mycelia of the fungi, two replicates for each fungus were submerged with a CuSO$_4$ solution. After reaching the desired size (5 – 8 cm), fungi were exposed to naphthalene (10 ppmV) and benzene (9000 ppmV). Concentrations of naphthalene and benzene and their possible degradation products were measured using PTR-TOF-MS after two weeks.

When comparing naphthalene concentrations for the fast-growing epiphytic fungi with the no fungus media controls no significant differences were observed (**Figure 13A** and **C**). A lower trend in naphthalene concentration was observed for the epiphytic fungus *Lophiostoma* sp. AT37, submerged in CuSO$_4$ solution growing on solid Kimura medium (**Figure 13B**) compared to the no fungus media-control. Benzene degradation by the selected fungi could not be determined because the spiked benzene concentrations were too high and could not be reliably measured by the PTR-TOF-MS.
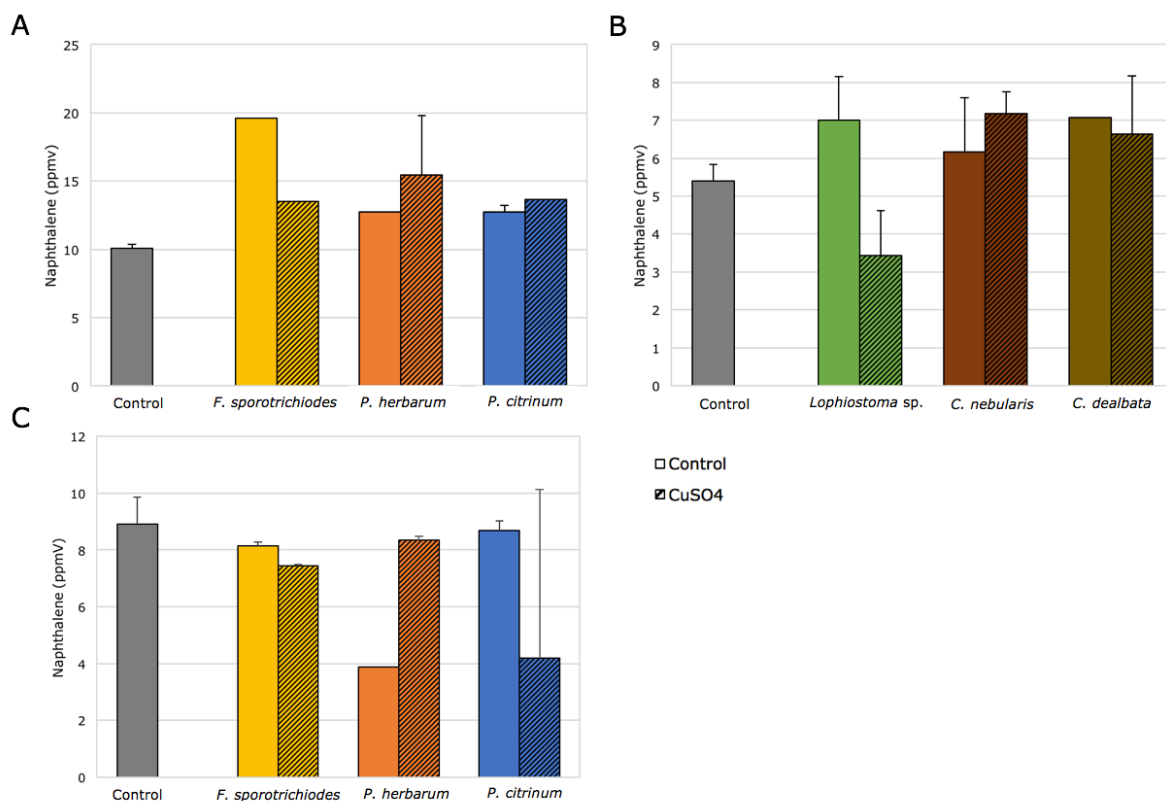
Figure 13. Naphthalene degradation by epiphytic fungi. The naphthalene concentration (ppmV) measured using PTR-TOF-MS is shown for (A) the fast-growing epiphytic fungi and (B) the slow-growing fungi in the Erlenmeyers growing on Kimura medium and (C) the fast-growing fungi in the Erlenmeyers with Ingestad medium containing 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose. No fungus media-controls are indicated in gray.

1.32 FDA ASSAY

To assess the metabolic activity of the fungi, tested for naphthalene degradation, a FDA assay was performed. More specifically, to assess if the fungi, submerged with the CuSO$_4$ solution, were indeed metabolically inactive. Metabolic activity (**Figure 14**) was indeed significantly lower, after adding the CuSO$_4$ solution, for each of the fungi compared to the control condition. However, a low level of metabolic activity could still be detected.

Figure 14. Fluorescein diacetate hydrolysis assay performed with the fungi F. sporotrichiodes, P. herbarum and P. citrinum. Statistically significant differences between control and CuSO4 conditions per fungus are indicated after performing Student's t-test by ** (p ≤ 0.01).

1.33 GAS CHROMATOGRAPHY – MASS SPECTROMETRY

Naphthalene concentrations were determined, using GC-MS, two weeks after performing the PTR-TOF-MS measurements. The slow-growing fungi of the first batch, growing on Kimura medium and the fast-growing fungi of the second batch, growing on Ingestad medium were selected. It appeared that the naphthalene concentration for the epiphytic fungus *Lophiostoma* sp. AT37 (**Figure 15A**) exposed to the CuSO₄ solution was significantly lower than the control condition (P ≤ 0.05). This significant reduction in naphthalene concentration was also seen for the basidiomycete *C. nebularis* ST1. No significantly differences in naphthalene concentration were observed for the fungi *F. sporotrichiodes* AT11 and *P. herbarum* AT15. Though, a lower trend in naphthalene concentration is visible for *P. herbarum* AT15, especially for the CuSO₄ exposed condition.
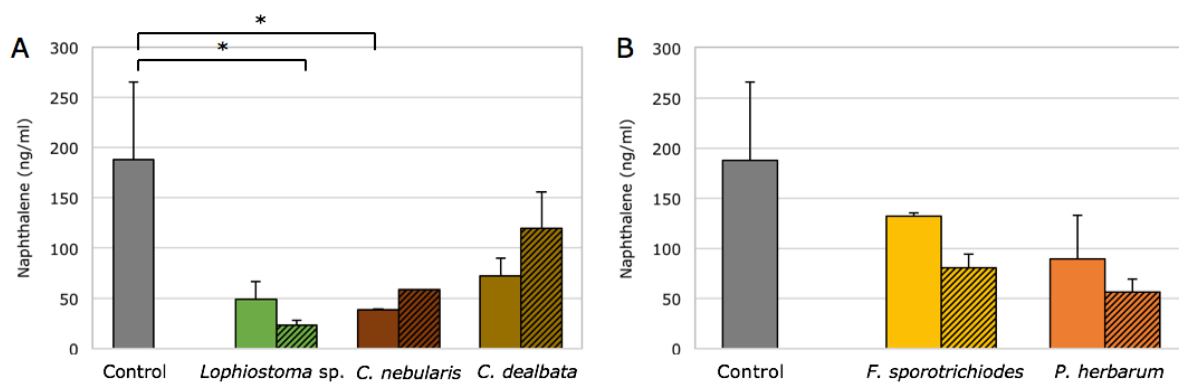
Figure 15. Gas Chromatography–Mass Spectrometry (GC-MS) measurement of naphthalene degradation by (A) the epiphytic fungus *Lophiostoma* sp. AT37 and the two fungi previously used as positive controls: *C. nebularis* and *C. dealbata*, growing on Kimura medium and (B) the epiphytic fungi F. sporotrichiodes AT11 and P. herbarum AT15, growing on Ingestad medium containing 2.5 g.L⁻¹ fructose and 2.5 g.L⁻¹ glucose, with shaded bars indicating the CuSO₄ exposed condition. No fungus media-controls are indicated in gray. Statistically significant differences are indicated after performing one-way ANOVA by: * (P ≤ 0.05).

1.34 PATHOGENICITY

Ivy plants were used to test pathogenicity of the most promising isolated epiphytic fungi. The fungi *F. sporotrichiodes* AT11, *P. herbarum* AT15, *P. citrinum* AT26 and *Lophiostoma* sp. AT37 were used to inoculate non-sterilized ivy plant leaves. Plants were hosted in a green house and watered regularly. Two weeks after inoculation with the fungi, ivy leaves were still looking healthy (**Figure 16**) in comparison to the not inoculated. No evidences of pathogenicity by the fungi could be detected after two weeks. The ivy plants in pots, one of them used for each different fungus, were still looking healthy and were growing.

Figure 16. Plant pathogenicity test of epiphytic fungi. The state of the ivy leaves at the beginning of inoculation (1) and after two weeks (2) are shown for (A) *F. sporotrichiodes* AT11, (B) *P. herbarum* AT15, (C) *P. citrinum* AT26 and (D) *Lophiostoma* sp. AT37.

DISCUSSION

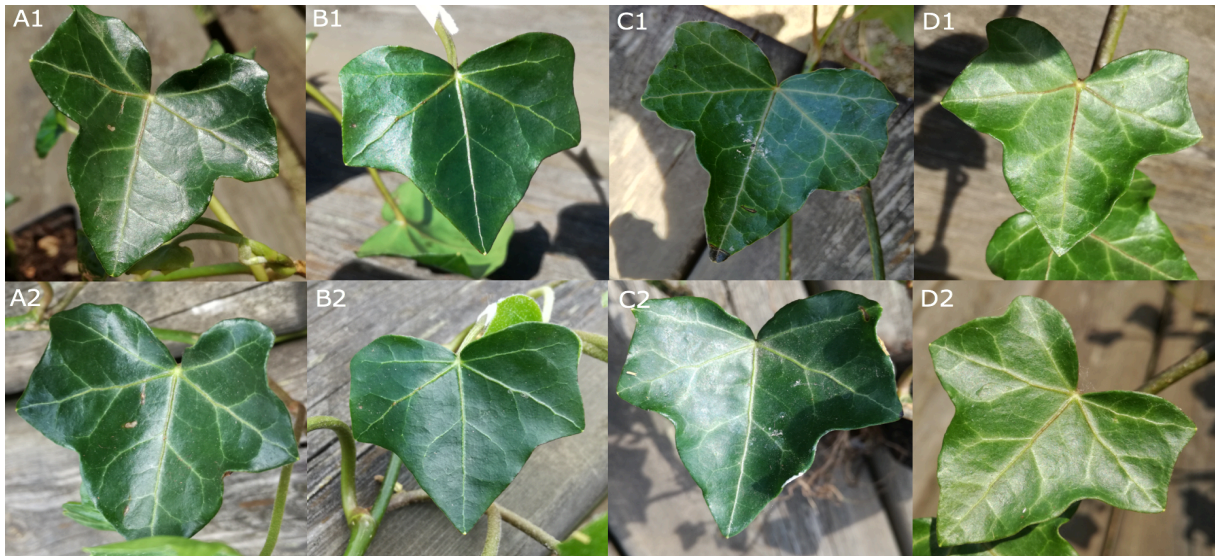Naphthalene (PAH) and benzene (MAH) are pollutants mainly present in the exhaust of diesel fueled cars and are therefore principal urban air pollutants. Exposure to these compounds is inevitable because of their ubiquitous presence, recalcitrant nature and persistence in the environment. Aromatic hydrocarbons pose a threat for human health and therefore, strategies to reduce these air pollutants and improve air quality are necessary. Plants and their microbiome may offer a cheap, sustainable and green alternative to traditional chemical and physical remediation approaches (23).

Our study hypothesized that the epiphytic fungi on leaves of hornbeam trees in a crude oil-contaminated area could degrade airborne AHs. We used Illumina Shotgun sequencing, Sanger sequencing, Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) to explore the taxonomical diversity and the AH degradation abilities of epiphytic fungal communities.

First, we taxonomically characterized the fungal communities living on the leaves of hornbeam trees. Second, we screened some representatives of the fungal community for their ability to degrade air-borne pollutants and production of catabolic enzymes involved in the degradation processes. Third, we tested the potential fungal pathogenicity on model ivy plants. This study presents for the first time insights about the fungi associated with hornbeam leaves and if and how some fungal species can assist the host in degrading naphthalene and benzene. Characterization of good degraders might pave the way for future effective bioremediation applications.

1.35 TOTAL AND CULTIVABLE EPIPHYTIC FUNGAL COMMUNITIES

Air pollution might play a role in shaping epiphytic fungal communities on hornbeam leaves. Our study shows how air pollution seems to significantly change the fungal community composition. We had significant evidence of the variability comparing fungal communities, living on leaves of hornbeam trees in two differently polluted areas: the crude oil-contaminated area of Bóbrka and the city center of Warsaw and the non-polluted Białowieża National Park.

ARISA DNA fingerprinting analysis showed that epiphytic fungal communities from Białowieża differed the most form the fungal communities in the phyllosphere of Warsaw and differed less from those in Bóbrka. In fact, fungi in the phyllosphere of Bóbrka could already have adapted to the polluted condition, more than the phyllospheric fungi in Warsaw. In Bóbrka, a massive crude oil contamination has already been present since the last glacial period (50). Another factor that might explain the community diversity among sites is that the petroleum site of Bóbrka is situated in a forest where there is a larger and more diverse community of trees, whereas in the city center of Warsaw, hornbeam trees are planted in parks and along roadsides. According to Jumpponen and Jones (27) reasons that might explain of the observed differences in fungal community structure include: genetic variation of the hosts, stand structure and size (single or clustered trees, rows, hedges…) properties of the soil and nutrient availability, differences in management. However, little is known about these factors influencing microbial communities and investigations are still ongoing. Although, Bobrka and Bialowieza are differently polluted areas they are still basically two forest-like

ecosystems and with some traits more similar in comparison to a city-center ecosystem. This might be a reason why fungal communities in Bialowieza and Bobrka are less different then when comparing Warsaw and Bialowieza. Even though, Bobrka is highly contaminated by hydrocarbons (more in the solid than in the air phase).

Characterization of total epiphytic fungal communities, using **Illumina Shotgun sequencing**, showed also an interesting trend of diversity in community composition among the different sites. Metagenome analysis and taxonomic identification of the phyllosphere fungal communities was performed using Kaiju, classified fungal genera with a relative abundance higher than or equal to 1.0% were investigated. The fungal phyllosphere communities of Białowieża and Bóbrka seemed more diverse than Warsaw ones when comparing relative abundances of classified fungal genera (≥ 1.0%). The phyllosphere of Warsaw was mainly dominated by the fungal genus *Aureobasidium*. This genus was also present in the phyllosphere of the other two sites, but to a lesser extent. Fungi belonging to the genus *Taphrina* were found in all three sites and the genus *Alternaria* in two sites: Białowieża and Warsaw. A study by Jumpponen and Jones (27) on the fungal phyllosphere communities in temperate regions indicated these three fungal genera as frequently present on leaves.

Most dominant colonizers of the hornbeam phyllosphere are bacteria. However, when the environment is contaminated by hydrocarbons, fungi seem to be more abundant in the polluted areas compared to the non-polluted area. The relative abundance of bacteria seemed lower in the polluted areas which might indicate that bacteria are more sensitive to air pollution. A study by Brighignal (34) also indicated that fungi are less affected or less sensitive to air pollution than bacteria. This might explain the trend of a higher relative abundance of fungi in the phyllosphere of the polluted areas compared to a lower abundance on leaves from the National Park of Białowieża.

Fungi were isolated and **cultivated from leaf wash samples** originating from the crude oil-contaminated area of Bóbrka and locations near the city center of Warsaw. The polluted areas were selected for isolation of fungi, because it was expected that these sites might harbor more fungi capable of degrading airborne pollutants (35). We mainly focused on the phyllosphere of the petroleum site of Bóbrka, because it was expected that on this site the chance of finding a fungus that could degrade AHs was high, due to the thousands of years of crude oil permeation through the soil. Even when sampling on this site the scent of the pollutants, volatile and semi-volatile organic compounds, was noticed in the air.

Metagenome analysis suggested that fungal communities, from all sampling sites, were dominated by fungi belonging to the phylum Ascomycota. Jumpponen and Jones (27) also observed a dominance of Ascomycetes in the phyllosphere of temperate regions. Remarkably, all fungi isolated and cultivated from leaf wash samples, belonged to the phylum Ascomycota, no basidiomycetes had been cultivated. Even no basidiomycetes were cultivated from the leaf wash samples from the site of Bóbrka, which had the highest relative abundance of basidiomycetes (43%) compared to the other sites. However, this might depend on the isolation and culturing methods used. Common media (MEA, Czapek dox) were used for cultivation, to obtain a fungal population as large as possible

for further experiments. According to Thorn (64), basidiomycetes are challenging to be isolated because ascomycetes can produce large numbers of spores which can outgrow basidiomycetes on traditional culture media.

Among the commonly present phyllospheric fungal genera, fungi belonging to the genera *Alternaria*, *Aureobasidium* and *Cladosporium* (27), were cultivated from leaf wash samples. Besides these, fungi of the genera *Fusarium*, *Lophiostoma*, *Penicillium*, *Phoma*, *Acremonium* and *Sarocladium* were also cultured.

Isolated epiphytic fungi were further investigated if they possessed the mechanisms expected to be used in the degradation of AHs and if they could indeed degrade these airborne pollutants. The fungi *Alternaria*, *Cladosporium* and *Sarocladium* were not selected for further experiments, after consulting with prof. dr. François Rineau, because of the pathogenic properties they can exert on plants and the adverse effects the can have on human health (74–76).

1.36 AH DEGRADATIVE ENZYMES AND METABOLITES.

We still have little understanding about which enzymes are present in epiphytic phyllospheric fungi to degrade aromatic hydrocarbons, or which other mechanisms are used (for example radical production). Therefore, the most important enzymes known to break down AHs in basidio- and ascomycetes, including laccase and peroxidase enzymes, were investigated. Laccase and peroxidase enzymes are excreted by fungal cells into the environment and have a low substrate specificity, which makes them suitable for degrading various organic pollutants including AHs (77).

Laccase enzymes, which are also called multicopper oxidases, catalyze a four-electron-reduction of molecular oxygen to water and simultaneously oxidize a substrate. By using this mechanism they are able to oxidize various substrates including AHs (78). **Peroxidases** including lignin peroxidases which contains an iron protoporphyrin IX in their catalytic center and manganese peroxidases. Are enzymes requiring hydrogen peroxide (H_2O_2) as electron accepting co-substrate to catalyze oxidation reactions. Lignin and Manganese peroxidases are the two main ligninolytic enzymes used by white-rot fungi known to degrade various organic compounds (77). Several ascomycetes are known to produces some of these peroxidases, however, these enzymes are only little studied in ascomycetes (79).

The epiphytic fungi *Lophiostoma* sp. appeared to have the highest enzyme activities for both laccase and peroxidase enzymes compared to the other isolated epiphytic fungi. Enzyme activities of 7.2 µU/L for laccase and a peroxidase enzyme activity of 7.49 µU/L were detected. The fungi *F. sporotrichiodes* AT11 and *P. herbarum* AT15 were also able to produce these enzymes. However, enzyme activities for these two fungi were lower.

The fungi C. nebularis, C. dealbata and Ganoderma sp. were used in the enzyme tests as positive controls. C. dealbata had a peak enzyme activity of 28.8 µU/L, which was comparable to the enzyme activity of C. nebularis with a peak activity of 26.4 µU/L. Ganoderma sp. had a laccase enzyme activity of 11.8 µU/L. A study by Torres presented evidence of laccase enzyme production by

Ganoderma sp. with enzyme activities of between 42.2 and 424.3 U/L. Laccase Enzyme activities tested here for Ganoderma sp. were lower.

Lower enzyme activities might be explained by the type of medium used to culture the fungi. Torres used the SB-U medium with sugarcane molasses as carbon source which induces ligninolytic enzyme production (80). Sugarcane molasses is a byproduct of sugar production, it contains nutrients necessary for fungal growth. Additionally, it contains phenolic acids and aromatic compounds derived from lignin that stimulate production of ligninolytic enzymes (42). The medium that was used in our experiments was the Kimura medium (20 g/L glucose, 5g/L peptone, 2 g/L yeast extract, 1 g/L KH_2PO_4 and 0.5 g/L $MgSO_4.7H_2O$). Enzyme production might have been higher if aromatic compounds were added to the medium as in the SB-U medium.

Besides the ligninolytic enzymes, production of **hydroxyl radicals** by fungi is a possible mechanism to degrade organic pollutants. Hydroxyl radicals are produced extracellularly by Fenton chemistry and redox cycling of quinones and are extremely powerful oxidants (81,82). Fungal hydroxyl radical production of the cultured epiphytic fungi was determined by spectrophotometrically measuring the oxidation of the substrate terephthalate to hydroxylterephthalate. The epiphytic fungi *F. sporotrichiodes* AT11 and *Lophiostoma* sp. AT37 produced significantly more hydroxyl radical compared to *P. citrinum* and *P. herbarum*. The basidiomycete *Ganoderma* sp. produced the highest amount of hydroxyl radicals. This might be explained by the fact that *Ganoderma* sp. lives on the bark of trees and uses these hydroxyl radicals to degrade lignin, which is a component of wood. However, if the phyllospheric fungi produce the hydroxyl radicals on leaves, concentration cannot be too high otherwise the plant tissue might be damaged because of oxidative stress. Though, plants and their microbiome have evolved in such a way that they are able to cope with oxidative stress (23,83).

1.37 TOLERANCE TO MAHs AND PAHs

Our results indicate that the fungus *Lophiostoma* sp. was affected by exposing it to naphthalene and benzene. After 11 days, net growth was reduced when exposed to the contaminants. Tough, it seemed more tolerant than the other epiphytic fungi that were tested. *F. sporotrichiodes* AT11 and *P. herbarum* AT15 were significantly more affected when exposed to naphthalene and benzene. Net growth of the fungi *F. sporotrichiodes* AT11 and *P. herbarum* AT15 after two days of exposure was remarkably increased but after five days the growth was affected and a decline in net growth rate was recorded. It seems that in the beginning the fungus could cope better with the hydrocarbon contamination but when pollution persists the growth decreased. Nevertheless, the fungi were still able to grow in the polluted environment, the growth was only slowed down.

Tolerance of *P. citrinum* to naphthalene and benzene could not be determined because there was not much fungal growth in both the exposed as well as the control condition. It seemed that the type of medium selected to conduct the experiment was not suitable to sustain growth of the fungus *P. citrinum*. The medium used to perform the tolerance test was solid Ingestad medium containing 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose. Which is a rather poor medium and represented better the environment on plant leaves (32).

The pigmentation of the fungi *F. sporotrichiodes* AT11 and *P. herbarum* AT15 was also altered after exposing them to naphthalene and benzene. *Fusarium sporotrichiodes* AT11 which produced bright pink to red pigments on the Ingestad medium with 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose, stopped producing these pigments after exposure to the contaminants. Pigmentation of the fungus *P. herbarum* AT15 was also reduced after exposure. The MAH and PAH tolerance test was performed using a rather nutrient poor medium and under natural day-night cycle to simulate better the environment these fungi live in. When this fungus was growing on nutrient rich MEA medium and not directly exposed to sunlight, *F. sporotrichiodes* AT11 did not produce these red pigments as much as on the rather nutrient poor Ingestad medium with 2.5 g.L^{-1} fructose and 2.5 g.L^{-1} glucose.

Pigments such as melanins (dark- brown pigments), carotenoids (orange red), lycopene (dark red) are usually produced by fungi as a protection mechanisms against environmental stress including: UV light, ionizing radiation, drought or oxidative stress (84,85). According to Pagano and Dhar (84), pigments can also be produced when less nutrients are available. The exposure of the fungus *F. sporotrichiodes* AT11 to sunlight and the limited nutrients might explain the bright red pigmentation. However, when stress conditions are too high, it might be that the fungal metabolism and consequently the secondary metabolites production including pigment production can be disrupted. Tis might explain the lack or reduction of pigmentation after exposure to naphthalene and benzene.

1.38 NAPHTHALENE DEGRADATION

The epiphytic fungi *F. sporotrichiodes*, *P. herbarum*, *P. citrinum* and *Lophiostoma* sp. were selected for the AH degradation experiment. These fungi were chosen because they seem to possess one or more of the mechanisms needed to degrade AHs, including: laccase and/or peroxidase enzymes and/or hydroxyl radical production.

It appears that for the epiphytic fungus *Lophiostoma* sp., when CuSO$_4$ solution was added, a lower trend in naphthalene concentration was measured compared to the control condition. The results from the GC-MS measurements, which were performed two weeks after the PTR-TOF-MS analysis, indicated a significantly lower naphthalene concentration compared to the control condition. Tis might indicate that more time is required for the fungus to degrade the pollutants. Only naphthalene concentrations could be measured using the PTR-TOF-MS, because benzene concentrations were too high.

We assessed the a-specific absorption of the contaminants in the mycelia of the fungi using a metabolically inactive replicate of the fungus. To make the fungi metabolically inactive, two of the four replicates were submerged with a CuSO$_4$ solution (300 mM). Copper is an essential element for growth and survival of the fungus. However, high copper concentrations are toxic. Copper can interact with nucleic acids, alter active sites of enzymes and oxidize components of the membrane (86). We chose for this fungicide above others because this compound is an inorganic compound and expected not to interfere with the measurements of the air samples using the PTR-TOF-MS and GC-MS.

After adding the CuSO$_4$ solution, we expected a higher concentration of the contaminants when measuring air samples taken from the closed Erlenmeyers. However, no such clear trend was observed. For some fungi, even lower naphthalene concentrations were measured after adding the CuSO$_4$ solution. Therefore, metabolic activity of the fungi was assessed by performing a FDA assay. The fast-growing fungi form the first batch, growing on the Kimura medium, were used to perform the assay. It appeared that metabolic activity was indeed significantly reduces but not completely. Therefore, in the future other techniques or compounds should be used to ensure metabolic inactivity such as high temperatures together with high pressure or exposure to UV radiation (86).

1.39 PLANT PATHOGENICITY TEST

No pathogenicity by the epiphytic fungi was seen after two weeks of inoculation on common ivy plants (*Hedera helix*). The most promising epiphytic fungi: *Lophiostoma* sp. AT37, *F. sporotrichiodes* AT11, *P. herbarum* AT15, *P. citrinum* AT26 were inoculated on the leaves of ivy plants. After two weeks, all leaves still looked healthy and no major differences were found among control ivy leaves and inoculated ones.

To further test possible pathogenicity of the fungi on the plants, the state and color of the leaves should be investigated over a longer period of time. Presence of fungal spores and invasiveness of the fungal hyphae into the plant surface and possibly into the plant cells could be assessed using general microscopic techniques and Scanning Electron Microscopy. Pathogenicity at a molecular level could also be verified by investigating the presence of pathogenicity genes in the genome of the fungi and the expression of these genes by reverse transcription Real Time PCR.

Further field experiments will be performed to assess AH degradation by fungi inoculated on plant leaves. The most promising epiphytic fungi will again be inoculated on ivy plant leaves. The leaves will be enclosed in Teflon bags and naphthalene and benzene will be spiked into the bags. After around three weeks, air samples from these bags will be taken and measured using PTR-TOF-MS. Concentrations of the spiked pollutants and their possible degradation products will be determined.

CONCLUSION

In our study, we have shed light on and increased the knowledge of the epiphytic fungal microbiome on hornbeam leaves. We compared epiphytic fungal communities from three differently polluted locations: the crude oil-contaminated area of Bóbrka, the city center of Warsaw and the National Park of Białowieża. Data obtained in this thesis suggests that air pollution plays a role in shaping the fungal communities inhabiting the phyllosphere of hornbeam leaves.

The cultivated epiphytic fungus *Lophiostoma* sp. was the most promising fungus, isolated from the phyllosphere of Bóbrka, to degrade airborne AHs. This fungus had the highest laccase and peroxidase enzyme activities and produced also hydroxyl radicals.

A possible AH degrader was characterized. In fact, *Lophiostoma* sp. AT37 showed interesting values of naphthalene degradation, measured using PTR-TOF-MS and GC-MS. These first results will be further verified and additional *in vitro* degradation tests as well as *in vivo* AH degradation tests in the field will be carried out. Though, this research is at its very beginning, this innovative seven-months investigation on phyllosphere fungi can be considered as a first step towards the developing of new bioremediation approaches for cleaner air.

REFERENCES

1.   Smith TM, Smith RL. Elements of Ecology. Pearson; 2009. 649 p.
2.   Kampa M, Castanas E. Human health effects of air pollution. Environ Pollut. 2008;151(2):362–7.
3.   Popescu F, Ionel I. Anthropogenic air pollution. In: Air Quality. 2010. p. 1–23.
4.   Nemery B, Hoet PHM, Nemmar A. The Meuse Valley fog of 1930: An air pollution disaster. Lancet. 2001;357(9257):704–8.
5.   Mosley S. SA NE M SC PL O E – C EO AP LS TE S M SC PL O E –. 1950;
6.   EEA. Air quality in Europe — 2016 report. 2016. 88 p.
7.   Costa DL, Amdur MO. Air pollution. Casarett Doull's Toxicol Basic Sci Poisons. 1996;857–882.
8.   Abdel-Shafy HI, Mansour MSM. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. Egypt J Pet [Internet]. 2015;25(1):107–23. Available from: http://www.sciencedirect.com/science/article/pii/S1110062114200237
9.   Haritash AK, Kaushik CP. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. J Hazard Mater. 2009;169(1–3):1–15.
10.   Brocco D, Fratarcangeli R, Lepore L, Petricca M, Ventrone I. Determination of aromatic hydrocarbons in urban air of Rome. Atmos Environ. 1997;31(4):557–66.
11.   Patnaik P. HYDROCARBONS, AROMATIC. In: A Comprehensive Guide to the Properties of Hazardous Chemical Substances. 2nd ed. John Wiley & Sons Publishers; 1999. p. 541–61.
12.   Westerholm R, Egeback KE. Exhaust emissions from light- and heavy-duty vehicles: Chemical composition, impact of exhaust after treatment, and fuel parameters. Environ Health Perspect. 1994;102(SUPPL. 4):13–23.
13.   Ravindra K, Sokhi R, Van Grieken R. Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation. Atmos Environ. 2008;42(13):2895–921.
14.   Lee B-K, Van Tuan V. Sources , Distribution and Toxicity of Polycyclic Aromatic Hydrocarbons (PAHs) in Particulate Matter. In: Air Pollution [Internet]. InTech; 2010. p. 26. Available from: http://www.intechopen.com/books/air-pollution/sources-distribution-and-toxicity-of-polyaromatic-hydrocarbons- pahs-in-particulate-matter%0AInTech
15.   Suess MJ. The environmental load and cycle of polycyclic aromatic hydrocarbons. Sci Total Environ. 1976;6(3):239–50.
16.   Wild SR, Jones KC. Polynuclear aromatic hydrocarbons in the United Kingdom environment: A preliminary source inventory and budget. Environ Pollut. 1995;88(1):91–108.
17.   Agency for Toxic Substances and Disease Registry (ATSDR). Public Health Statement: Polycyclic Aromatic Hydrocarbons (PAHs). AtsdrCdcGov [Internet]. 1995;6. Available from: http://www.atsdr.cdc.gov/toxprofiles/tp16-a.pdf
18.   National Toxicology Programme. Polycyclic Aromatic Hydrocarbons : 15 Listings. 2011.
19.   IARC. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Non-heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures. Iarc Monogr Eval Carcinog Risks To Humans. 2010;92:1–868.
20.   Boffetta P, Jourenkova N, Gustavsson P. Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes Control [Internet]. 1997;8(3):444–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9498904
21.   Armstrong B, Hutchinson E, Unwin J, Fletcher T. Lung cancer risk after exposure to polycyclic aromatic hydrocarbons: A review and meta-analysis. Environ Health Perspect. 2004;112(9):970–8.
22.   Green M, Righini E, Kassow J, Espin R, Lee E-K, Milani C. "Final warning" on Air pollution from the European Commission. Latham & Watkins LLP. 2017 Mar;
23.   Weyens N, Thijs S, Popek R, Witters N, Przybysz A, Espenshade J, et al. The role of plant-microbe interactions and their exploitation for phytoremediation of air pollutants. Int J Mol Sci. 2015;16(10):25576–604.
24.   Dzierżanowski K, Popek R, Gawrońska H, Sæbø A, Gawroński SW. Deposition of Particulate Matter of Different Size Fractions on Leaf Surfaces and in Waxes of Urban Forest Species. Int

J Phytoremediation [Internet]. 2011;13(10):1037–46. Available from: http://www.tandfonline.com/doi/abs/10.1080/15226514.2011.552929

25. Vorholt JA. Microbial life in the phyllosphere. Nat Rev Microbiol [Internet]. 2012;10(12):828–40. Available from: http://www.nature.com/doifinder/10.1038/nrmicro2910

26. Lindow SE, Brandl MT. Microbiology of the Phyllosphere MINIREVIEW Microbiology of the Phyllosphere. Appl Environ Microbiol. 2003;69(4):1875–83.

27. Jumpponen A, Jones KL. Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate Quercus macrocarpa phyllosphere. New Phytol. 2009;184(2):438–48.

28. Cordier T, Robin C, Capdevielle X, Desprez-Loustau ML, Vacher C. Spatial variability of phyllosphere fungal assemblages: Genetic distance predominates over geographic distance in a European beech stand (Fagus sylvatica). Fungal Ecol. 2012;5(5):509–20.

29. Newton AC, Fitt BDL, Atkins SD, Walters DR, Daniell TJ. Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. Trends Microbiol [Internet]. 2010;18(8):365–73. Available from: http://dx.doi.org/10.1016/j.tim.2010.06.002

30. Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL. Contribution of Fungal Loline Alkaloids to Protection from Aphids in a Grass-Endophyte Mutualism. Mol Plant-Microbe Interact [Internet]. 2000;13(10):1027–33. Available from: http://apsjournals.apsnet.org/doi/abs/10.1094/MPMI.2000.13.10.1027

31. Louisa Howard. SEM Images - epiphytic microorganisms [Internet]. [cited 2017 May 20]. Available from: http://remf.dartmouth.edu/Malus_domestica_apple_SEM_P2/

32. Müller T, Ruppel S. Progress in cultivation-independent phyllosphere microbiology. FEMS Microbiol Ecol. 2014;87(1):2–17.

33. Kembel SW, Mueller RC. Plant traits and taxonomy drive host associations in tropical phyllosphere fungal communities [1]. Botany [Internet]. 2014;92(4):303–11. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/cjb-2013-0194

34. Brighignal L, Gori2 A, Gonnelli2 S, Favilli F. The influence of air pollution on the phyllosphere microflora composition of Till andsia leaves (BromeHaceae). Rev Bio! Trop [Internet]. 2000;48(23):51–17. Available from: www.ucr.ac.cr%5Cnwww.ots.ac.cr%5Cnwww.ots.duke.edu

35. Kannangara S, Sirisena DM. Aromatic Hydrocarbon Degrading Fungi Inhabiting the Phyllosphere of Ornamental Plants on Roadsides of Urban Areas in Sri Lanka. J Bioremediation Biodegrad [Internet]. 2016;7(1):1–7. Available from: http://www.omicsonline.org/open-access/aromatic-hydrocarbon-degrading-fungi-inhabiting-the-phyllosphere-ofornamental-plants-on-roadsides-of-urban-areas-in-sri-lanka-2155-6199-1000328.php?aid=68677

36. Yang H, Liu Y. Phytoremediation on Air Pollution. In: Phytoremediation of Air Pollutants: A Review. 2007. p. 293–314.

37. Dela Cruz M, Christensen JH, Thomsen JD, Müller R. Can ornamental potted plants remove volatile organic compounds from indoor air? — a review. Environ Sci Pollut Res. 2014;21(24):13909–28.

38. Tuomela M, Hatakka A. Oxidative Fungal Enzymes for Bioremediation [Internet]. Second Edi. Vol. 6, Comprehensive Biotechnology, Second Edition. Elsevier B.V.; 2011. 183-196 p. Available from: http://dx.doi.org/10.1016/B978-0-08-088504-9.00370-6

39. Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, et al. Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev. 2008;32(6):927–55.

40. Bamforth SM, Singleton I. Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. J Chem Technol Biotechnol. 2005;80(7):723–36.

41. Novotný Č, Svobodová K, Erbanová P, Cajthaml T, Kasinath A, Lang E, et al. Ligninolytic fungi in bioremediation: Extracellular enzyme production and degradation rate. Soil Biol Biochem. 2004;36(10):1545–51.

42. Torres G, León AMM, Rineau F, Alonso LLL, María I, López S, et al. Diversity of ligninolytic enzymes and their genes in strains of the genus Ganoderma: applicable for biodegradation of xenobiotic compounds? Front Microbiol. 2017;

43. Marco-Urrea E, García-Romera I, Aranda E. Potential of non-ligninolytic fungi in bioremediation of chlorinated and polycyclic aromatic hydrocarbons. N Biotechnol.

2015;32(6):620–8.

44. Doddapaneni H, Yadav JS. Microarray-based global differential expression profiling of P450 monooxygenases and regulatory proteins for signal transduction pathways in the white rot fungus Phanerochaete chrysosporium. Mol Genet Genomics. 2005;274(5):454–66.

45. Worrall JJ, Anagnost SE, Zabel RA. Comparison of wood decay among diverse lignicolous fungi. Mycologia. 1997;89(2):199–219.

46. Liers C, Arnstadt T, Ullrich R, Hofrichter M. Patterns of lignin degradation and oxidative enzyme secretion by different wood- and litter-colonizing basidiomycetes and ascomycetes grown on beech-wood. FEMS Microbiol Ecol. 2011;78(1):91–102.

47. Harms H, Schlosser D, Wick LY. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol [Internet]. 2011;9(3):177–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21297669

48. Mark H. Brand. Carpinus betulus, European Hornbeam, Common Hornbeam - Plant Database - University of Connecticut [Internet]. University of Connecticut. [cited 2017 May 23]. Available from: http://www.hort.uconn.edu/plants/detail.php?pid=83

49. Gilman EF, Watson DG. Carpinus betulus "Fastigiata": "Fastigiata" European Hornbeam [Internet]. University of Florida. [cited 2017 May 23]. Available from: https://edis.ifas.ufl.edu/st119

50. Muzeum Przemysłu Naftowego i Gazowniczego im. Ignacego Łukasiewicza in Bóbrce. Oil field | BÓBRKA [Internet]. [cited 2017 May 24]. Available from: http://bobrka.pl/en/oil-field-in-bobrka/

51. WEJMUTKA Białowieża Biodiversity Academy. Bialowieza Primeval Forest .: bialowieza biodiversity academy, Wejmutka:. bia�?owieża, forest, biodiversity, workshop, nature, hotel [Internet]. [cited 2017 May 24]. Available from: http://www.wejmutka.pl/eng/bialowieza-primeval-forest.php

52. Kurpas D, Mroczek B, Brodowski J, Urban M, Nitsch-Osuch A. Does Health Status Influence Acceptance of Illness in Patients with Chronic Respiratory Diseases? Advs Exp Med Biol Respir. 2015;6(October 2014):57–66.

53. Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J, Nazaret S. Characterization of Bacterial and Fungal Soil Communities by Automated Ribosomal Intergenic Spacer Analysis Fingerprints: Biological and Methodological Variability. Appl Environ Microbiol. 2001;67(10):4479–87.

54. Menzel P, Ng KL, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun [Internet]. 2016;7:11257. Available from: http://www.nature.com/doifinder/10.1038/ncomms11257

55. Keegan KP, Glass EM, Meyer F. MG-RAST, a Metagenomics Service for Analysis of Microbial Community Structure and Function. In 2016 [cited 2017 May 25]. p. 207–33. Available from: http://link.springer.com/10.1007/978-1-4939-3369-3_13

56. Thom C, Church MB. The Aspergilli. 1926.

57. Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Van Gijsegem F. Alcaligenes eutrophus CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol. 1985;162(1):328–34.

58. LURIA SE, BURROUS JW. Hybridization between Escherichia coli and Shigella. J Bacteriol. 1957;74(4):461–76.

59. Bergersen FJ. Nitrate reductase in soybean root nodules. Biochim Biophys Acta [Internet]. 1961;52(1):206–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/0006300261909222

60. SCHLEGEL HG, KALTWASSER H, GOTTSCHALK G. [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies]. Arch Mikrobiol [Internet]. 1961 [cited 2017 May 29];38:209–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13747777

61. Stevens V. The structure of phyllospheric microbial communities and their role in phytoremediation of air pollution. Hasselt University; 2016.

62. Bushnell LD, Haas HF. The Utilization of Certain Hydrocarbons by Microorganisms. J Bacteriol. 1941;41(5):653–73.

63. Ingestad T, Kähr M. Nutrition and growth of coniferous seedlings at varied relative nitrogen addition rate. Physiol Plant [Internet]. 1985;65(2):109–16. Available from: http://dx.doi.org/10.1111/j.1399-3054.1985.tb02368.x

64. Thorn RG, Reddy CA, Harris D, Paul EA. Isolation of aprophytic basidiomycetes from soil. Appl Environ Microbiol. 1996;62(11):4288–92.

65. Teerapatsakul C, Abe N, Bucke C, Kongkathip N, Jareonkitmongkol S, Chitradon L. Novel laccases of Ganoderma sp. KU-Alk4, regulated by different glucose concentration in alkaline media. World J Microbiol Biotechnol. 2007;23(11):1559–67.

66. Claiborne A, Fridovich I. Purification of the o-Dianisidine Peroxidase from Escherichia coli B. J Biol Chem. 1979;254(10):4245–52.

67. Page SE, Arnold WA, McNeill K. Terephthalate as a probe for photochemically generated hydroxyl radical. J Environ Monit [Internet]. 2010;12(9):1658. Available from: http://xlink.rsc.org/?DOI=c0em00160k

68. Brilli F, Gioli B, Ciccioli P, Zona D, Loreto F, Janssens IA, et al. Proton Transfer Reaction Time-of-Flight Mass Spectrometric (PTR-TOF-MS) determination of volatile organic compounds (VOCs) emitted from a biomass fire developed under stable nocturnal conditions. Atmos Environ [Internet]. 2014;97(August):54–67. Available from: http://dx.doi.org/10.1016/j.atmosenv.2014.08.007

69. The HDF Group - Information, Support, and Software [Internet]. [cited 2017 Jun 8]. Available from: https://www.hdfgroup.org/

70. Lindinger W, Hansel A, Jordan A. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) medical applications, food control and environmental research. Int J Mass Spectrom Ion Process [Internet]. 1998;173(3):191–241. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0168117697002814

71. Warneke C, Van Der Veen C, Luxembourg S, De Gouw JA, Kok A. Measurements of benzene and toluene in ambient air using proton-transfer-reaction mass spectrometry: Calibration, humidity dependence, and field intercomparison. Int J Mass Spectrom. 2001;207(3):167–82.

72. Cappellin L, Karl T, Probst M, Ismailova O, Winkler PM, Soukoulis C, et al. On quantitative determination of volatile organic compound concentrations using proton transfer reaction time-of-flight mass spectrometry. Environ Sci Technol. 2012;46(June):2283–90.

73. Adam G, Duncan H. Development of a sensitive and rapid method for the measurement of total microbial activity using ¯uorescein diacetate (FDA) in a range of soils. Soil Biol Biochem. 2001;33:943–51.

74. Gnanamanickam SS, Mew TW. Interactions between Sarocladium oryzae and stem attacking fungal pathogens of rice. Plant Soil. 1991;138(2):213–9.

75. Peternel R. Atmospheric concentrations of Cladosporium ORIGINAL ARTICLES. 2017;(February 2004).

76. Mamgain A, Roychowdhury R, Tah J. REVIEW Alternaria pathogenicity and its strategic controls. Res J Biol. 2013;1:1–9.

77. Tuomela M, Hatakka A. Oxidative Fungal Enzymes for Bioremediation [Internet]. Second Edi. Vol. 1, Comprehensive Biotechnology. Elsevier B.V.; 2011. 183-196 p. Available from: http://dx.doi.org/10.1016/B978-0-08-088504-9.00370-6

78. Collins PJ, Kotterman MJJ, Field J a, Dobson a DW. Oxidation of anthracene and benzo[α]pyrene by laccases from Trametes versicolor. Appl Environ Microbiol. 1996;62(12):4563–7.

79. Lopez MJ, Vargas-García M del C, Suárez-Estrella F, Nichols NN, Dien BS, Moreno J. Lignocellulose-degrading enzymes produced by the ascomycete Coniochaeta ligniaria and related species: Application for a lignocellulosic substrate treatment. Enzyme Microb Technol. 2007;40(4):794–800.

80. Manzano AM, Torres G, Gonzalez A, Banguela A, Ramos-Gonzalez PL, Valiente PA, et al. Role of Laccase isoenzymes in textile dye decolorization and diversity of laccase genes from Ganoderma weberianum (B-18). J Appl Sci Environ Sanit. 2013;8(4):237–42.

81. Krueger MC, Bergmann M, Schlosser D. Widespread ability of fungi to drive quinone redox cycling for biodegradation. FEMS Microbiol Lett. 2016;363(11):1–5.

82. Rineau F, Roth D, Shah F, Smits M, Johansson T, Canbäck B, et al. The ectomycorrhizal fungus Paxillus involutus converts organic matter in plant litter using a trimmed brown-rot mechanism involving Fenton chemistry. Environ Microbiol. 2012;14(6):1477–87.

83. Cross CE, Van der Vliet A, Louie S, Thiele JJ, Halliwell B. Oxidative stress and antioxidants at biosurfaces: Plants, skin, and respiratory tract surfaces. Environ Health Perspect. 1998;106(SUPPL. 5):1241–51.

84. Pagano MC, Dhar PP. Fungal pigments: An overview. Fungal Biomol Sources, Appl Recent Dev. 2015;(Isaac 1994):173–89.

85. Medentsev AG, Arinbasarova AY, Akimenko VK. Biosynthesis of naphthoquinone pigments by fungi of the genus Fusarium. Appl Biochem Microbiol. 2005;41(5):503–7.

86. Cervantes C, Gutierrez-Corona F. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol Rev [Internet]. 1994;14(2):121–37. Available from: https://academic.oup.com/femsre/article-lookup/doi/10.1111/j.1574-6976.1994.tb00083.x

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