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**Bioremediation of soils contaminated
by polycyclic aromatic hydrocarbons**

Bachelor's thesis

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Declaration

I declare, that the Bachelor Thesis Bioremediation of soils contaminated by polycyclic aromatic hydrocarbons is my own work and all the sources I cited in it are listed in Bibliography. As an author I also declare, that I have not violated any copyrights.

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Bioremediation of soils contaminated by polycyclic aromatic hydrocarbons

Summary

The aim of this work was to summarise characteristics and origin of 16 USEPA polycyclic aromatic hydrocarbons, which were labeled as substances with high risk and mostly potential carcinogens. Effects on health have been discussed, with the focus on agricultural and livestock production, through which these pollutants can travel to human diet. In the last part of the literature overview, various remediation techniques, especially bioremediation, were described in detail. Former and recent research on this topic was mentioned and taken into account.

In the experimental part, we established an outdoor experiment in natural conditions, to test the ability of plants along with fungal inoculate to remediate PAH contaminated soil. 32 pots of contaminated soil, mixed with *Pleurotus ostreatus* inoculated wood chips were used for planting of *Zea mays*, which then grew for 120 days, while periodically watered and weeded. There were 8 variants of this setup, each of 4 repetitions, with differences in use of fungal inoculated substrate and size of wood chips used for growing *P. ostreatus*. Half of the pots were planted with maize to compare effectivity of fungal degradation alone and its cooperation with phytoremediation. Referencial samples were also established, with only bare soil without substrate or maize. After 120 days, plant and fungal biomass were harvested, samples of soil were collected and analysed for the content of single PAHs, using gas chromatography.

Results showed that the most efficient option was the combination of plants and fungi, inoculated on wood chips of 30-50 mm size. Average degradation of 16 PAHs for this variation was 37 %, while low and medium molecular weight of PAHs was degraded more efficiently. This shows that high molecular weight PAHs are more resistant, which assents to previous research. These results were considered as very good, since bioremediation is an environmentally friendly and accessible method. Plant and fungal biomass contained little or no PAHs (the most abundant compound was naphthalene), which is very positive result. It showed that grown on contaminated soil, crops do not accumulate pollutants and could be further used as food or feed for animals.

Key words: PAHs, *Pleurotus ostreatus*, phytoremediation, ligninolytic fungi

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1 Introduction

Polycyclic aromatic hydrocarbons are widely spread compounds which are toxic for the environment and organisms, ranging from the simple ones like naphthalene, phenanthrene, to more complex PAHs like benzo(a)pyrene. In total, there are many compounds labeled as PAHs but only a small number of most common PAHs have been extensively studied. PAHs originate naturally for example during forest fires, formation of crude oils or by some plants and bacteria, but also during industrial processes made by human. PAHs are proven carcinogens, cause damage of internal organs as well as respiratory issues. Not only humans but also farm-animals can get affected, which can lower quality of livestock products like meat, milk or eggs, as PAHs can be bioaccumulated in the organs of living organisms. Many efforts have been made to remove PAHs from the environment however there is no quick and easy way to degradate them - PAHs have very low water solubility and easily get bound to lipid tissues which is also why they are very resistant to degradation. Rate of degradation always depends on environmental conditions, mixture of PAHs present during degradation and their concentration. Above physical and chemical degradation, biodegradation has shown to be the most effective way. Recent studies have shown that bacteria and algae can help remove PAHs from polluted water, whereas plants and fungi are useful in the fields with contaminated soil. The experimental part of this thesis focuses on degradation by plants (*Zea mays*) and fungi (*Pleurotus ostreatus*) together and put into comparison with bare soil samples, in a soil polluted by PAHs, with duration of 120 days. Harvested maize and collected fungal biomass as well as the soil were then analysed for PAHs content by gas chromatography.

2 Objectives of the work

1. The purpose of the literature part was to introduce PAHs – their occurrence, origin, physical and chemical characteristics, along with their environmental routes. Toxicity to humans and animals and their possible effect on crops and livestock products were discussed in detail.

2. To characterize the basic principals and possibilities of PAH remediation in soil and to compare conventional methods with environmental friendly methods leading to elimination of PAH, or lower its impact on environment, including human and farm-animals/crops.

3. The aim of the experimental part was to test the combination of phytoremediation and fungal degradation when remediating soil PAHs in field conditions, using *Zea mays* L. and *Pleurotus ostreatus* Jacq., comparing their both separate and cooperative effects.

4. The final objective was to state whether crop biomass tends to bioaccumulate pollutants and therefore poses a risk as a human food or feed for farm-animals, where it can store up in various products and in many cases find way to human diet.

3 Literature overview

3.1 Origin and occurrence of PAHs

Polycyclic aromatic hydrocarbons (PAHs) present a group of over a hundred organic compounds. They have been extensively studied due to their negative environmental and health impact even at low exposure (Bostrom et al. 2002). They are ubiquitous in the environment and their resistant properties contribute to their ability to travel through various media like air, soil or water and the bioaccumulate in organic tissues (Baklanov et al. 2007).

3.1.1 Chemical formation

The general principle of PAH formation is saturation of hydrocarbons under low-oxygen conditions, in presence of pyrolysis and pyrosynthesis. High temperatures cause to break bonds in the molecules, forming free radicals, which then condensate and form PAHs. Such reaction is shown in Figure 1 (Ravindra et al. 2007). Depending on reaction factors like speed and temperature, different PAHs form (Wiersum 2008).

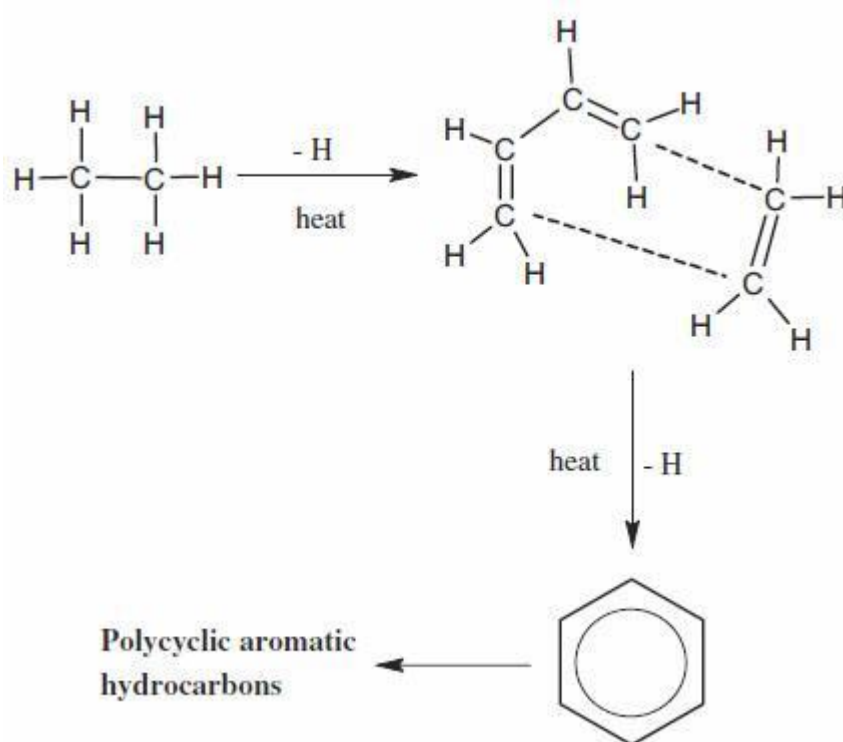


Fig.1 – Transformation of ethane into PAHs by pyrosynthesis (Ravindra et al. 2007)

Sources of PAH formation are generally divided in natural and anthropogenic, where anthropogenic origin is the most significant in recent decades (Wilcke 2000).

PAHs are formed by several ways, some of which are more common than others. The way in which these compounds form affects their amount and kind.

First is pyrogenic – when organic substances encounter high temperatures with no or low oxygen, pyrolysis takes place. Temperature needed for this process starts only at 350 °C and

reaches up to 1200°C. Examples of intentional pyrolysis are distillation of coal into coke or breaking petroleum into simpler hydrocarbons. However, another source of pyrolysis is incomplete combustion of fossil fuels in traffic or wood during forest fires, which is happening at much bigger scale and is unintentional. In fact, incomplete combustion is known to be the biggest source of PAHs (Adams et al. 2015). In 2019, Mohseni-Bandpei et al. 2019 measured that the temperature influences the formation of PAHs, especially their amount. As an experiment, they used medical waste, which is commonly destructed by pyrolysis. Also, the smaller the particles for pyrolysis were, the less PAHs were formed.

PAHs can form after millions of years under even lower temperatures, 100-150 °C, in processes that are called petrogenic. Example of these substances are crude oils. Because of their extensive use, they often get into environment by oceanic oil spills and underground tank leaks and also in small amounts by any car or sort of machine running on fossil fuels. (Adams et al. 2015).

The last way in which PAHs are being produced, is the least known. They can form biologically by some bacteria and plants (Abdel-Shafy & Mansour 2015).

3.1.2 PAHs divided by origin

All above mentioned PAH sources can also be listed as natural or anthropogenic. Forest fires and other natural cases of incomplete combustion, crude oil formation or bacterial/algal processes being natural sources, while oil spills, fossil fuel burning and some industrial combustion processes belonging to anthropogenic sources (Abdel-Shafy & Mansour 2015). Release of PAHs to some extent depends on season, when in winter there is more biomass burnt, producing pollutants, also cold weather contributes to better accumulation of these substances. In summer the major source are industrial processes, not so much dependant on the season (Han et al. 2020).

Another source of PAH formation by human, which should not be forgotten, is heat-treatment of food. The temperature, time, certain ingredients and preparation method all influence which PAHs and in what amount they are formed (Darwish et al. 2019). PAHs are also common part of various edible oils and flavourings, like gravies for instant noodles etc. They may not be in the food from the factory, but often form due to inappropriate storage (Gong et al. 2019).

Some studies take smoking into account, as it is proven to be carcinogenic and contains PAHs as well as many other unhealthy substances and can affect research results in for example PAH occupational hazards. Singh et al. (2018) did an analysis of lung cancer risk among coal/coke industry workers, where many of them smoked. This lead them to a conclusion that they couldn't satisfactory present their results as the smoking could be affecting them greatly.

In 2010, estimated anthropogenic emissions of the 16 EPA PAHs were 621 tonnes. When only focusing on benzo(a)pyrene, which was 3.23 tonnes of the amount, the emission originated in following sources: urban combustion 76 %, industrial combustion 6.7 %, traffic 4.3 %, metal production 3.4 %, waste incineration 1.0 % and other sources 8.4 % (Kim et al. 2013).

3.1.3 PAH deposition and transport

the greatest concentrations above urban areas from where they spread (Kim et al. 2013). After being produced by vehicular/petroleum emission, newly formed PAHs get emitted to the atmosphere or accumulate in street dust, from which they get easily ingested. (Ghanavati et al. 2019) While in the air, PAHs are present either as a vapor or as solids, bound to small particles. Hydrophobic organic substances like PAHs have a relatively low vapor pressure which causes them to bind easily to atmospheric particulates, although there are differences between vapor pressures of particular PAHs, meaning they occur in different concentrations in vapor and sorbet to particles (more PAHs are present in vapor phase the higher their vapor pressure is), all this is also dependant on the atmosphere moisture and temperature (Lawal 2017).

It is estimated that over 40 million people, only in Europe, are exposed to over-limit levels of pollution (Baklanov et al. 2007). While in the air, particles of PAHs which are small enough, when deposited onto surfaces, can damage mechanical devices like engines and affect their performance, as well as for example degrade cultural materials and art (Miguel et al. 2005).

PAHs then get deposited from the atmosphere through precipitation to the soil or water, where they can become bioavailable and get metabolised and accumulated by microorganisms and plants. PAHs do not tend to penetrate deep into the soil thanks to their physical properties (Wild & Jones 1994). Some amount of pollutants gets discharged into soil and water as a leftover waste from industrial processes that has not been safely liquidated (Aydin et al. 2017).

Many studies suggest that soils that are near to urban and industrial areas are most contaminated. Before human influence, content of naturally present PAHs ranges between 1-10 ng/kg, while soils near contamination sources contain at least 10 times higher levels of PAHs (Wilcke 2000).

PAHs are also present in sediments. Sources like ship traffic, oil spills or urban runoff cause water pollution from where PAHs get slowly deposited into sediments, where they are tightly bound and immobile, thanks to their hydrophobic nature and low solubility. The example of highly polluted sediments are river bottoms and swamps down the stream from urban areas, or ocean floors near frequent ship transport routes (Tam et al. 2000; Brown & Peake 2005). Sediments with large size fraction tend to accumulate more PAHs than fine sediments like clay or silt, which is a rule that also appears with soils – simply put, media which is less permeable does not let through PAHs as easily (Wang et al. 2001). An interesting media for contents of PAHs is snow, which is more easily contained and measured than air and doesn't undergo as many bioremediation processes as soil, therefore shows a clear record of air pollution (from which the snow got contaminated) throughout time (Izvekova et al. 2018). Tong et al. (2018) measured amounts of PAHs that get ingested by hand-to-mouth activities and concluded that for children aged 6-12 years, the amounts are nonnegligible. Children often have bad hygienic habits and can ingest indoor dust or outdoor soil by their hand-to-mouth and object-to-mouth incidents, where there can be a high contamination, especially in urban areas.

Concerning effects of PAHs on the environment and organisms, they are proven to be toxic substances. Its toxicity depends on several factors, but they are known to be more harmful under UV light. PAHs are especially toxic to aquatic organisms and birds, causing them tumors, immunity/reproduction issues and more. Organisms living in contaminated soil such as small

invertebrates are not highly affected, unless the contamination level is really high (Abdel-Shafy & Mansour 2015).

When bound to air particles, PAHs can travel long distances in the atmosphere. They have been found on places far from the source of their creation like tropical forests and polar regions. Also their global concentration has increased in last 30 years (Kuppusamy et al. 2016).

3.2 Properties of major PAHs

One of the most distinctive characteristic of PAHs is their resistance. They have high boiling and melting points which is also why they are solids, along with very low aqueous solubility - they are hydrophobic. The more rings a PAH has, the less water soluble it is and gets more resistant to oxidation and reduction. On the other hand, because of their high lipophilicity PAHs are highly soluble in organic solvents. The age of the compound also affects its resistance as well as co-existence with other pollutants, like heavy metals or other hydrocarbons (Bamforth & Singleton 2005).

Among over a hundred polycyclic aromatic hydrocarbons, scientists have used lists of most common and significant PAHs, to make their research more comparable and less complex. Most common list that has been used since 1970 was made by USEPA (United States Environmental Protection Agency) and it consists of 16 PAHs (Andersson & Achten 2015). It is still used til this day in many studies (Vernoux et al. 2011), some other sources even only show 12 PAHs (Miguel et al. 2005). It has been discussed recently, that when only focused on PAHs from this list, results can be incomplete and not representative of the whole group. Some other lists have been introduced, for example 40EnvPAHs, ment to better evaluate environmental toxicity (Wise et al. 2014). However, the 16 PAH USEPA list is still considered very useful especially because all 16 PAHs are available for purchase and laboratory testing (Andersson & Achten 2015).

Below are listed 16 USEPA PAHs, their short characteristics, structure and rational chemical formulas. Each PAH is shown on matching picture.

Naphtalene (Fig. 2)

The most simple PAH consisting of two rings. Appears as white solid of aromatic smell. When ingested, naphtalene causes acute hemolytic anemia, damages liver and inflicts neurological damage. It has also been classified as probable carcinogen by IARC (Agoun-Bahar et al. 2019). Naphthalene makes about 10 % of coal tar by weight – it is the most abun dant PAH in coal tar. It can also be derived from petroleum, which is then more pure than naphthalene separated from coal tar (Kairbekov et al. 2019).



Fig. 2 - naphthalene

Acenaphthylene (Fig. 3)

An ortho- and peri-fused hydrocarbon of three rings. Its appearance is solid of yellow color, with no fluorescence (Griesbaum et al. 2000). Its toxicity has been defined by no-observed-effect-level in rats, which has been calculated to 4 mg/kg/day (Tanabe et al. 2017). Acenaphthylene occurs in coal tar, in contents of about 2 % and is industrially produced from acenaphthene by gas phase dehydrogenation (Griesbaum et al. 2000).

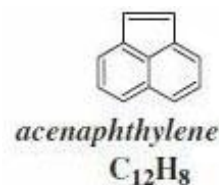


Fig. 3 - acenaphthylene

Acenaphthene (Fig. 4)

This three ringed polycyclic aromatic hydrocarbon is linked to previously mentioned acenaphthylene as they can be synthesized of each other by hydrogenization/dehydrogenation. They have similar properties except acenaphthylene is more saturated (Sribala et al. 2019). Coal tar contains about 0.3 % of this PAH. It is often used to produce plastics, dyes, fungicides and insecticides which has led to high accumulation in the soil in current days. Extensive research has been made to degrade acenaphthene and remove it from the environment (Mallick 2019).



Fig. 4 - acenaphthene

Fluorene (Fig. 5)

Or sometimes called 9H-fluorene is a simple PAH of three aromatic rings, which has a similar appearance to naphthalene. Even though it is classified as PAH, it has no aromatic properties, but acts as a weak acid. Fluorene can be obtained by dehydrogenating diphenylmethane or as a part of coal tar (Griesbaum et al. 2000). It is used as a parent compound - derived substances find their use as pharmaceuticals or to prepare dyes. Polyfluorene polymers are electroluminescent (Shin et al. 2006).

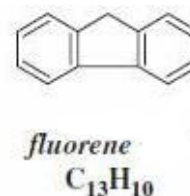


Fig. 5 - fluorene

Phenanthrene (Fig. 6)

Consists of three benzene rings, appears as transparent or pale yellow solid. It is used for production of dyes, pesticides and in pharmaceuticals. Phenanthrene is more easily degraded than other PAHs and therefore can be used as a model compound for research (Fanesi et al. 2018).

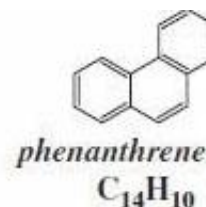
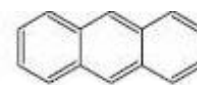


Fig. 6 - phenanthrene

Anthracene (Fig. 7)

This PAH, also of three rings, is colorless but emits blue fluorescence under the exposure of UV light. Its major source remains coal tar with 1,5 % of content (Griesbaum et al. 2000). When ingested or when skin is under direct exposure, it can cause inflammation. Long term effects could include higher cancer risk and mutations, even though only proven in animals so far (Holoubek, 1996). In 2010, anthracene was added to the list of substances of extreme concern according to the REACH authorisation (Šuta 2010).

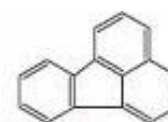


anthracene
 $C_{14}H_{10}$

Fig. 7 - anthracene

Fluoranthene (Fig. 8)

A four ringed PAH named after its fluorescence under UV light, appears as colorless or pale yellow solid. It was originally found in coal tar pitch in weighing content of few percent (Griesbaum et al. 2000). Fluoranthene was identified as a carcinogen, by the International Agency for Research on Cancer, after being tested on newborn mice (Favre & Powell 2013). Fluoranthene was also listed as a substance of very high concern, because of its toxic and persistent properties (classified by European Commission in 2019).



fluoranthene
 $C_{16}H_{10}$

Fig. 8 - fluoranthene

Pyrene (Fig. 9)

Pyrene has been under a focus for a long time and has served as model compound to investigate PAHs. It consists of four rings and as any other representative of the group, it is hydrophobic and bioaccumulative and resistant in the environment (Klankeo et al. 2009). Pyrene itself has not been proven carcinogenic but it can trigger other PAHs like benzo(a)pyrene to act out their carcinogenic properties. Also, metabolised pyrene produces quinones, which are much more harmful than the parent compound (Wei et al. 2017).

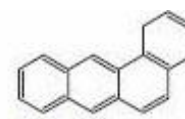


pyrene
 $C_{16}H_{10}$

Fig. 9 - pyrene

Benz(a)anthracene (Fig. 10)

A four-ring PAH listed as a priority chemical due to its probable carcinogenicity. It has high acute toxicity, bioaccumulative character and is chemically stable (Rachna et al. 2019)



benzo[a]anthracene
 $C_{18}H_{12}$

Fig. 10 – benz(a)anthracene

Chrysene (Fig. 11)

Consists of four rings and appears as golden/yellow solid. From its color, the name is derived (chrysos - “gold” in Greek). Chrysene is often bound to tetracene which is orange/yellow and cannot be easily separated. However, pure chrysene is colorless. This PAH is suspected carcinogen although not proven to be toxic alone, as it is usually mixed with other more dangerous PAHs. It is a constituent of tobacco smoke, also found in coal tar and creosote (Ojha et al. 2019).

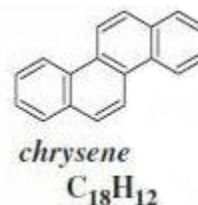


Fig. 11 - chrysene

Benzo(b)fluoranthene (Fig. 12)

This aromatic hydrocarbon consists of 5 rings and appears as yellow powder or even small needles. It is primarily found in gasoline exhaust, tobacco and cigarette smoke, coal tar, and products of pyrolysis. Benzo(b)fluoranthene is reasonably anticipated to be a human carcinogen (NCI Thesaurus 2020).

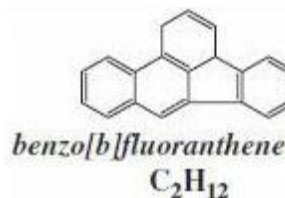


Fig. 12
– benzo(b)fluoranthene

Benzo(k)fluoranthene (Fig. 13)

Similar to benzo(b)fluoranthene, this PAH has 5 fused rings and appears as pale yellow solid. It is produced by the incomplete combustion of organic matter and is primarily found in gasoline exhaust, cigarette smoke, coal tar, and industrial bi-products. As well as benzo(b)fluoranthene, it does not have practical use, only as a research material. Benzo(k)fluoranthene is reasonably anticipated to be a human carcinogen (NCI Thesaurus 2020).

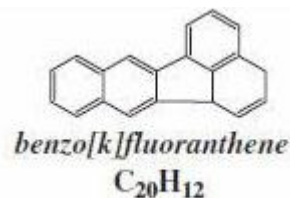


Fig. 13 –
benzo(k)fluoranthene

Benzo(a)pyrene (Fig. 14)

BaP consists of five aromatic rings and it is the most frequently studied PAH. It contributes to higher carcinogenic risk to humans – classified by IARC as a human carcinogen. BaP is used as a marker to measure how polluted ambient air is and therefore whether it has a carcinogenic risk. (Guerreiro et al. 2016)



Fig. 14 – benzo(a)pyrene

Benzo(g,h,i)perylene (Fig. 15)

PAH consisting of six aromatic rings, naturally present in coal tar. Benzo(a)perylene is white or colorless crystalline solid. As a high molecular weight PAH it is very resistant and remains in the environment for a long time. This substance is used only for analytical work and has no practical use. It has been proven that benzo(g,h,i)perylene is carcinogenic and teratogenic, and has bioaccumulative properties (Haničincová & Válek 2014).



Fig. 15 – benzo(g,h,i)perylene

Dibenz(a,h)anthracene (Fig. 16)

A crystalline, carcinogenic aromatic hydrocarbon consisting of five fused benzene rings. Dibenz(a,h)anthracene is primarily found in gasoline exhaust, tobacco smoke and coal tar. This substance is used only for research purposes to induce tumorigenesis. Dibenz(a,h)anthracene is a mutagen and is reasonably anticipated to be a human carcinogen (NCI Thesaurus 2020).

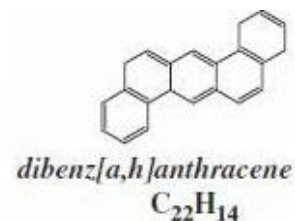


Fig. 16 – dibenz(a,h)anthracene

Indeno(1,2,3-cd)pyrene (Fig. 17)

This six-ring hydrocarbon has been confirmed to have toxic, carcinogenic and mutagenic properties. It has high molecular weight which causes its highly hydrophobic and resistant nature (Ojha et al. 2019).

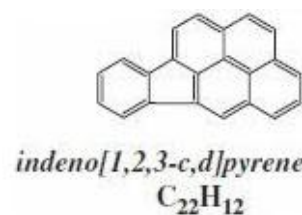


Fig. 17 – indeno(1,2,3-cd)pyrene

3.3 Uptake of PAH by plants

PAHs are lipophilic, with very low solubility in water. They easily accumulate in lipid tissues or are sorbed to plant surface from where they get in our diet. PAHs in vapor state can also travel through stomata on leaves. In general, plants with simply larger surface area contain more surface-bound PAHs. Even though much PAHs can be removed only by washing the plant (those bound to surface particles), those that are incorporated in lipid tissues (for example in carrots) are almost impossible to eliminate (Srogi 2007; Onyedikachi et al. 2019).

Not all plants will absorb PAHs, also not all PAHs are easily taken in for accumulation. Only compounds with small molecules and low molecular weight can travel in transpiration stream through which they find lipid tissues to bind to (2-3 ring PAHs), heavier and larger molecules (3-6 ring PAHs) only sorb onto outer layer of roots. A study on uptake of POP (persistent organic pollutants) by plants, using willow, poplar, maize and sunflower supports this fact. The plants were fertilized and grown on polluted soil for two years after which they were harvested and tested for pollutant content. PAHs were most efficiently absorbed by roots of willow. Phenanthrene accumulated in plant parts above ground, pyrene was found in roots (Kacálková & Tlustoš 2010). Another study showed that warming (warming in laboratory

conditions was to simulate effects of global warming on the environment) makes PAHs more accessible to plants, both from the soil and the air. Chen et al. (2019) used spinach for this experiment, and proceeded to a conclusion that successful uptake by plants can help bioremediate PAHs, but has a high risk of ingestion of such polluted plants/crops.

Effects that PAHs have on plants vary. They can stimulate or inhibit their growth, due to their impact on seed germination and ability to affect building and growth of tissues and therefore whole plant biomass. (Afegbua & Batty 2018) In the same article, Afegbuy & Batty (2018) published a study where *Meticago sativa* showed an increase in biomass yield while *Lolium perenne* produced a lower amount of biomass in comparison to the control, when exposed to PAH mixture. Aranda et. al (2013) made an experiment to test whether PAHs in the soil can inhibit root growth of plants in symbiosis with fungi. They used 4 different PAHs as contaminants, for plant they used carrot (*Daucus carota L.*), which they had colonised with *Rhizophagus custos*. After 7 weeks, they analysed root colonization, PAH contents and root dry weight and found out, that mycorrhizal fungi stimulated the root growth by 30-40 %. Effect of PAHs was interesting – anthracene had no effect at all, but phenanthrene and dibenzothiophene (aromatic heterocycle containing sulphur) inhibited root growth by 60 % with a concentration of 60 μ M. When twice the concentration, root growth was reduced by 80 % - 92 %.

Some organisms like lichens can be used as a source of information about PAH bioaccumulation. Herzig et al. 2019 compared samples of lichens in Switzerland between 1995 and 2014, for amount of present pollutants. They found out that in time, concentration went down by 40-80 % on average in all site categories. This shows that regulations about these substances had a positive effect in general, however near road traffic and industrial sites concentration in some cases even increased.

3.4 Risks of PAHs for animals and humans

PAHs have various effects on health, for humans as well as for animals. Because of their lipophilicity, they tend to accumulate in adipose (lipid) tissues, in organs. They can get into the system either by ingestion, inhalation or through skin (Bamforth & Singleton 2005). From there, they get gradually released into the system.

Risk for humans is not only from direct exposure, but also from contaminated livestock products like meat, eggs or milk. Even though PAHs are not easily uptaken by plants, animals like cattle or poultry can be exposed with accidental ingestion of soil which can be polluted (Feidt et al. 2013). Jurnanz et al. (2012) measured that when dairy cows are not given suitable grazing conditions, they can ingest up to 1.5 kg of soil per day. Ugochukwu et al. (2018) analysed soil samples of six pastures for cattle, of which three were highly polluted and presented a significant risk for grazing cattle.

The most discussed health risk of PAHs is their proven carcinogenicity. They even were the first discovered carcinogens present in the environment (Haritash & Kaushik 2009). Due to their chemical structure, given by angular arrangement of the molecules, they bind to nucleotides, and also produce DNA-damaging byproducts like diol epoxides, quinones or PAH radical cations (Gao et al. 2018). Darwish et al. (2019) presented in their study of PAH contents

in heat-treated meat, that higher concentrations induce mutagenesis and cause higher production of ROS (reactive oxygen species) in cells.

Seven PAHs are most likely carcinogenic for human, according to USEPA. These are benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene and indeno(1,2,3-cd)pyrene (Gao et al. 2018). Statistically, workers exposed to high dosages of PAHs at work places like coke production, commercial kitchens, aluminium smelters, iron and steel foundaries, coal mines, chimney sweeping, road paving or professional drivers exposed to diesel engine exhaust are in a high risk of various types of cancer, mainly lung or skin cancer (Boffetta et al. 1997; Singh et al. 2018).

This however does not only include professionals working in hazardous conditions, but people living in urban areas with intensive traffic and high air pollution are at risk of chronic respiratory, cardiovascular or neurological diseases (Guilbert et al. 2018). In European population it is estimated that in areas with measurable pollution levels (60 % of population) there are around 370 new cases of lung cancer every year, connected to PAH polluted environment (Guerreiro et al. 2016). Han et al. (2020) placed their study in China, where they compared various PAH sources and their impact on health. They presented that PAHs released caused 15.198 excess lifetime cancer cases in total. In 2019, Ghanavati et al. studied street dust in Abadan, Iran, for the presence of heavy metals and PAHs and their effect on local population health. According to their research, these substances contribute to higher risk of cancer, caused both by dust ingestion/inhalation and dermal contact.

Besides occupational hazard and traffic pollution, the major route for PAHs through which they enter human body, is food consumption, especially in modern countries (Domingo & Nadal 2015). In 2010, average dietary PAH intake for a standart man was 6.72 µg/day, mostly through inhalation and cooked food (Martorell et al. 2010). The food does not only obtain PAHs through cooking, contamination via polluted soil should not be underestimated. Since PAHs can sometimes accumulate in vegetables and other crops, they can then be found even in raw food, that has not been burned, fried or in any way processed (Ray et al. 2012; Onyedikachi et al. 2019). Smoking, as a source of PAHs, also needs to be mentioned. Smoking can directly lead to lung cancer, by breathing in PAHs from cigarette smoke. According to the International Agency for Research on Cancer (in 2004), 90 % lung cancer deaths are linked to smoking (Wang et al. 2012).

Depending on PAH concentration and lenght of exposure, effects can be acute or chronic. General acute effects depend on PAH concentration and way of entry into the organism. Symptoms are acute sickness like nausea, vomiting and diarrhea, eye irritation. High levels of PAHs can also cause skin irritation and inflammation (these effects are known to be caused by anthracene, benzo(a)pyrene and naphtalene, which can also cause alergic reactions) (Kim et al. 2013).

Chronic results of long-term exposure to PAHs were previously mentioned on the example of workers in contaminated work place. Besides high risk of cancer, they include various defects of immune system, damage of organs like liver, kidney and lungs, asthma can develop. Other effects are teratogenicity and genotoxicity (causing mutations) (Lawal 2017). There were also studies focusing on effects PAHs have on children, who don't have fully developed respiratory, immune or reproduction systems and can be inhibited in their growth when influenced by polluted environment, leading to permanent disablement. This touches

mostly children between 2-3 years (Umeh et al. 2019). Prenatal exposure can lead to hyperactivity and other negative neurodevelopment (Oliviera et al. 2019). Zhang et al. (2019) measured levels of PAHs in primary schools, where children spend many hours per day. In two cases, concentration of BaP exceeded limits regulated by USEPA, especially in winter, when PAH levels are generally higher due to combustion for heating.

As mentioned before, PAHs can occur in oil-rich plant parts as well as on its surface (from where the contaminant can get washed away) Such food can also get contaminated during transport, when not sufficiently sealed (Kacálková & Tlustoš 2010).

However, even more severe source of PAHs in food is food processing and cooking, like smoking, grilling and frying. These processes fulfil the perfect conditions for creation of PAHs. In raw foods, content of PAHs ranges between 0.01-1 ug/kg, whether in smoked meat for example, levels of PAH content reached 200 ug/kg.

Another thing are PAHs in drinking water. The level of contamination is usually low (below 1 ng/l), depending on water pipe and storage tanks coating (asphalt or coal tar coating raises PAH levels) (Abdel-Shafy & Mansour 2015)

PAHs that get transported to fresh water by for example precipitation can then be bound to marine sediments where water filtering and sediment-dwelling organisms get in contact with them. Organisms like mussels and oysters filter large amounts of water, therefore can accumulate PAHs that later get in our diet. Also, animals that feed on these organisms contain PAHs too – like fish or squid. Speciale et al. (2018) conducted a research about blue mussel in benzo(a)pyrene polluted waters and their potencial risk for humans when consumed. They found a significant accumulation ability. When introduced to human circulating cells, organic extracts from contaminated mussels decreased rate of survival of these cells.

To summarize, major exposure routes of PAHs for human are smoked and grilled food, smoking (burning tobacco generates PAHs) and air pollution. Soils act as long term reservoirs of PAHs and it is crucial to be aware of current contamination when using soil for agriculture. All contaminated parts of the environment affect us and it is important to keep finding ways of PAH degrading everywhere in the environment (Srogi 2007).

3.5 Remediation of soils contaminated by PAHs

PAHs are considered to be persistent pollutants and therefore not easy to remove from the environment. When not intentionally using any bioremediation technique, they naturally „decompose“ with help of soil/water microbes and plants, but at very slow pace. Study shows that in first year since contamination, the degradation progresses at the fastest pace, later gradually slowing down (Harmsen & Rietra 2018). There are known several ways to remove or degrade PAHs present in the environment, varying greatly, depending on the environment and PAHs that need removal.

Further described ways to actually degrade PAHs show that the most efficient and cheapest way is probably biodegradation, which shows the most elegant results (Sakshi et al. 2019).

3.5.1 Physical, chemical degradation

Contaminated soils can be excavated from the site, taken away and then washed through with mixtures of water and co-solvents. The soil can also undergo other treatments, described below. Overall, these strategies categorized as physical-chemical degradation are expensive and often non-ecological. (?)

Processes involving the affect of light radiation or heat are another physical process of degradation. They occur naturally through sunlight (on the surface of contaminated soils and water) as well as intentionally mostly under laboratory conditions. Soils with low hydraulic permeability can be treated with electrokinetic remediation, using the principal of electromigration – low intensity current run through soil transports ionic pollutants (Kuppusamy et al. 2016).

Chemical degradation occurs when PAHs get oxidized by various reagents. Most well known are Fenton's reagent (Fe(II)-H₂O₂) and ozone (Gan et al. 2009). The fact that they are both toxic for microorganisms (Fenton's reagent is used with hydrogen peroxide) causes that further natural remediation is much slower and balance in the soil must therefore be re-established (Flotron et al. 2004). It is advisable to re-establish these soils with planting low-maintenance plants and trees, like willows, since the soil is usually still unsuitable for agricultural uses (Vervaeke et al. 2001). On the other hand, using proper oxidants in well decided doses can help to catalyse degradation of highly persistent PAHs like benzo(a)pyrene, which can further be degraded by bacteria with high effectivity. This method of pre-oxidation however needs well knowledge of the site to dose the oxidants correctly (Xu et al. 2018).

3.5.2 Bioremediation

Remediation by plants as well as bacterial and fungal remediation are collectively called bioremediation. In comparison with other methods of remediation, bioremediation has several advantages. It is eco-friendly, sustainable and shows great results (Sakshi et al. 2019).

When using organisms to alter the chemistry of the soil or water, a symbiosis can be established, where fungi support bacteria which can further support any plants/crops growing there, by increasing availability of nutrients with their metabolism (Aydin et al. 2017).

The precondition for bacteria, plants or fungi to be able to degrade persistent contaminants is their catabolic activity in using these xenobiotics for mineralization, as an energy source (Acevedo-Sandoval et al. 2018). Microorganisms and plants can either be added to the soil/water, which is called bioaugmentation. Biostimulation means supporting them with nutrients and better conditions for growth (Treu & Falandysz 2017).

Important factors that affect at which rate the PAHs are degraded involve the concentration of pollutant, its bioavailability and character of the site at which the degradation takes place, like moisture and temperature. Also, other chemicals and nutrients that are present, play an important role. They establish conditions for oxidation/reduction (electron acceptors like nitrate, sulfate, oxides of manganese and iron) and support microbial growth. All these characteristics always have to be taken into account, since they have a strong impact on results of any measured case (Adams et al. 2015). Bioavailability of PAHs in soil is to some extent influenced by other substances present. Ukalska-Jaruga et al. (2019) observed connections

between soil organic matter (SOM) fractions and PAHs. SOM fractions, which affected the bioavailability the most, were humins and black carbon. These fractions effectively increased retention and persistence of PAHs, making them less degradable.

Gomez-Eyles et al. (2010) found out that earthworms, important inhabitants of soil, increase PAH mobility by over 40 %, which is another factor that can be taken into account directly on the field.

Single PAHs have always different degradation rates, molecules with more benzene rings are generally more difficult to eliminate than those that are more simple. In recent years, the research has come so far that removing PAHs of up to 4 rings in the molecule is overall successful, whether benzo(a)pyrene, a five-ring compound present in coal tar, has gradually gathered focus when resisting even microorganisms that are usually effective at degradation (Samanta et al. 2002).

3.5.2.1 Phytoremediation

Phytoremediation means PAH degradation by photosynthesising organisms, mostly green plants.

Green plants can either help stabilize contaminants in the soil so they no longer hold an environmental risk, or they can remove the pollutants in various ways. However, the levels of pollution must be manageable for a plant or else it will not thrive and effectively remediate the soil (Cunningham et al. 1995). To some extent, plants can cope with bad conditions by releasing root exudates like amino acids, organic acids or carbohydrates. Since contaminated soils can often also lack one or more nutrients, plants tend to shift their conditions by these rhizodeposits, able for example to attract supportive bacteria or increase nutrient solubility and bioavailability (Carvalhais et al. 2010).

Plants that are capable of not only accumulation but also degradation of PAHs are many species of grasses and legumes, even trees (Mueller & Shann 2006). They can be used on large fields with surface contamination where other degradation methods would not be as suitable or environment friendly. Besides that, plants always co-exist with other organisms like bacteria and fungi, also able to remove pollutants. Enzymes secreted off of plant roots were identified as dehalogenase, nitroreductase, peroxidase, and laccase. These enzymes catalyse reactions in soil, leading to PAH changes. Since these enzymes get released into the soil and function outside of the plant, they can operate even after the plant died or has been harvested (Haritash & Kaushik 2009). Overall, planted soils show higher degradation of PAHs than soils with no plants by 30-40 % (Reilley et al. 1996).

A study by Han et al. (2016) supports this conclusion. They used ryegrass for phytoremediation of petroleum hydrocarbons. Their content in the soil was reduced by 30.34 %. The study also shows that adding biochar (which should for example increase soil fertility or water retaining capacity) does not contribute to pollutant removal, it actually had an opposite effect, inhibiting growth of ryegrass and therefore lowering the effectivity of degradation processes. Nonetheless, using ryegrass successfully helps to remediate petroleum hydrocarbon contaminated soils. Another study uses four Korean plant species in 80-day experiment. At the end, over 99 % of phenanthrene was degraded and 77-94 % of pyrene, compared to 99 % and 69 % in unplanted soil (Lee et al. 2007). Using willow (*Salix viminalis* L. 'Orm') has also proven

to be effective. In 1.5 years, the willow helped to degrade organic contaminants like PAHs and mineral oils and accumulate heavy metals from soil. However, in this work PAHs have shown higher degradation in the unplanted soil, while contents of mineral oils and heavy metals have decreased. The author states the reasons for this result were unclear (Vervaeke et al. 2002). The work of Bandowe et al. (2019) shows how beneficial it is to use plant mixtures for degradation. More diverse combination of plants supports soil bacteria more effectively and lowers PAH content faster.

Species of plants that are submerged in water can be useful when degrading PAHs in freshwater sediments. A study by (He & Chi 2017) focuses on removal of phenanthrene and pyrene by aquatic plants *Hydrilla verticillata* and *Vallisneria spiralis* in a 108-day period. Both species successfully degraded the two PAHs. *V. spiralis* by 85.9 % and 79.1, *H. verticillata* by 76.3 % and 64.6 %, respectively. Using plants to help stabilize or degrade and remove pollutants shows a great potential. Besides degradation, they add nutrients into soil and provide surface coverage, further improving soil quality. However, this strategy has many disadvantages, first of which are different results in greenhouse experiments and on the field. Reasons of these differences are not always discovered. Another disadvantage is relatively low speed of remediation as well as sensitivity to pollution level – at some point plants stop growing, when heavily polluted. Phytoremediation shows the best potential when combined with other remediation techniques, on soils suitable for plant growth (Gerhardt et al. 2008).

Another way to cope with high toxicity for plants when contamination levels are high is altering the plant composition, meaning using favorable species of plants that grow well together and support bacterial development in the soil. In experiment by Xie et al. (2018), there were used bristle grass and alfalfa separately, and in a mixture. When grown separately, growth of both plant species was significantly inhibited by the contaminant while when grown together, all aspects of growth, bacterial activity and degradation rates were improved.

3.5.2.2 Bacterial degradation

Bacterial degradation has been studied the most extensively. When PAHs are bioavailable for bacteria, their activity in degradation can be very high, even up to total removal of pollutants. In 2019, Feizi et al. used bacterium *Bacillus kochii* to bioremediate phenanthrene-contaminated soil, also testing the influence of soil salinity. For the phenanthrene concentration of 50 mg/kg with 1.5 % salinity, the degradation was 98 % efficient. With raising salinity, degradation efficiency went lower.

Bacteria are readily accepting PAHs as their energy source by the rule – lower number of aromatic rings gets digested more easily than more complex compounds. Soils with higher contamination also possess higher numbers of bacteria and show higher degradation activity than soils with lower concentration of pollutants (Cerniglia 1993).

Since different PAHs have different aqueous solubility, some get more easily bioavailable than others, depending on their molecular weight. Also, the time for which PAHs remain in the soil also has effect on their desorption speed.

The mechanism through which bacteria degrade pollutants differs for aerobic and anaerobic bacteria. Aerobic processes which are characteristic for soil environment are shown in Figure 18. They start at oxidation of aromatic ring/s, through several intermediates, to carbon

dioxide and water or PAH metabolites. (Bamforth & Singleton 2005) Many studies have been focused on finding bacterial genes responsible for PAH degradation, to control catabolic pathways during degradation more precisely and to be able to effectively assume its metabolites (Habe & Omori 2014). An interesting way to effectively use bacteria for soil remediation is using genetically modified strains, to enhance their ability of degradation. Sometimes they allow complete degradation, for example redesigned *Pseudomonas putida*, which is able to metabolise benzene, toluene and p-xylene with no metabolic intermediates. The downside of this approach are ethical aversions towards GMO, as well as insufficient data that distinguish between the degradation impact of GMO bacteria on their own and whole bacterial consortium of the soil, because of this results often remain unclear (Adams et al. 2015).

The area where bacteria and roots of plants meet, the rhizosphere, holds the perfect conditions for PAH degradation, in fact, a bigger part of the degradation happens there. Enzymes, secreted by plant roots make PAHs more bioavailable and also bacteria make nutrients in the soil more available for intake by plants, making all present organisms more prosperous. This again proves how beneficial it is to combine degradation strategies (Kotoky et al. 2017). Combining more bacterial strains into a consortium has been also proven beneficial. Vaidya et al. (2018) used 3 different bacterial strains to degrade chrysene, which is a very persistent PAH difficult to mineralize completely. The consortium effectively degraded and mineralized chrysene even in presence of other PAHs and heavy metals.

Lastly, both cyanobacteria and eucaryotic algae (green, red, brown algae) are capable of PAH removal, in aquatic environment, by photoautotrophic reactions (Cerniglia 1993). Even though algae are one of major aquatic organisms involved in bioremediation, not many studies have been carried out in comparison with bacteria or fungi. Their activity is strongly dependant on the type and intensity of light (gold, white or UV-A light). Metabolic routes also vary depending on the light (Ghosal et al. 2016).

3.5.2.3 Fungal degradation

A degradation by fungi is cost-effective and environmentally friendly, as well as other bioremediation techniques (Li et al. 2012). Fungi, contrary to bacteria, do not use PAHs as their main carbon and energy source, they rather degrade PAHs into more simple and un toxic compounds as a side effect of their metabolism. Both ligninolytic and non-ligninolytic fungi are capable of PAH oxidation, though ligninolytic fungi are much more effective and most of the research has been focused on them (Cerniglia 1993). There has been some research where soil fungi operated under very-low-oxygen conditions, using PAHs as a sole carbon source (Silva et al. 2008). However the amount of strains which are able to grow under these extreme conditions is limited (Aydin et al. 2017).

High humidity, ideal temperature and low pH are also factors affecting how fast and whether at all will be fungal enzymes active, varying slightly among different species of fungi (Tuor et al. 1995). Their strongest advantage, making them often more suitable for degradation than just bacteria or plants, is the ability to access soil pores with their mycelium, growing underground as one big organism able to relocate sources and "solve" more complex issues like obstacles in the soil or local lack of nutrients (Treu & Falandysz 2017).

Non-ligninolytic fungi such as Zygomycetes and Ascomycetes have shown to be able to degrade some highly persistent PAHs (benzo(a)pyrene), more effectively than bacteria. They grow well at neutral pH which makes them a good option for field usage. However their metabolic routes are not well known. Not many studies were focused on their metabolites and their possible effects and still need further research (Marco-Urrea et al. 2015).

Ligninolytic fungi can be divided in brown rot, soft rot and white rot fungi (Rabinovich et al. 2003), which are the only ones able to deconstruct lignin – an irregular and thick material. They use highly unspecific enzymes, which are also capable of degrading PAHs – enzymes such as lignin peroxidase, laccase or manganese peroxidase (Aydin et al. 2017; Haritash & Kaushik 2009). Laccase works as a catalyst for the initial reaction, causing the formation of quinones, which are ideally further oxidised by peroxidase, then mineralized (Pozdnyakova et al. 2018). These enzymes are extracellular, which means fungi can easily access pollutants with low bioavailability. The soil is often enriched with media containing fiber used as feed by fungi, to support its growth when focusing on fungal remediation, like milled wood or for example bran flakes (Pickard et al. 1999). The ways in which PAHs are deconstructed – lignolytic and nonlignolytic (fungal and bacterial) degradation – are shown in a simple diagram, Figure 18.

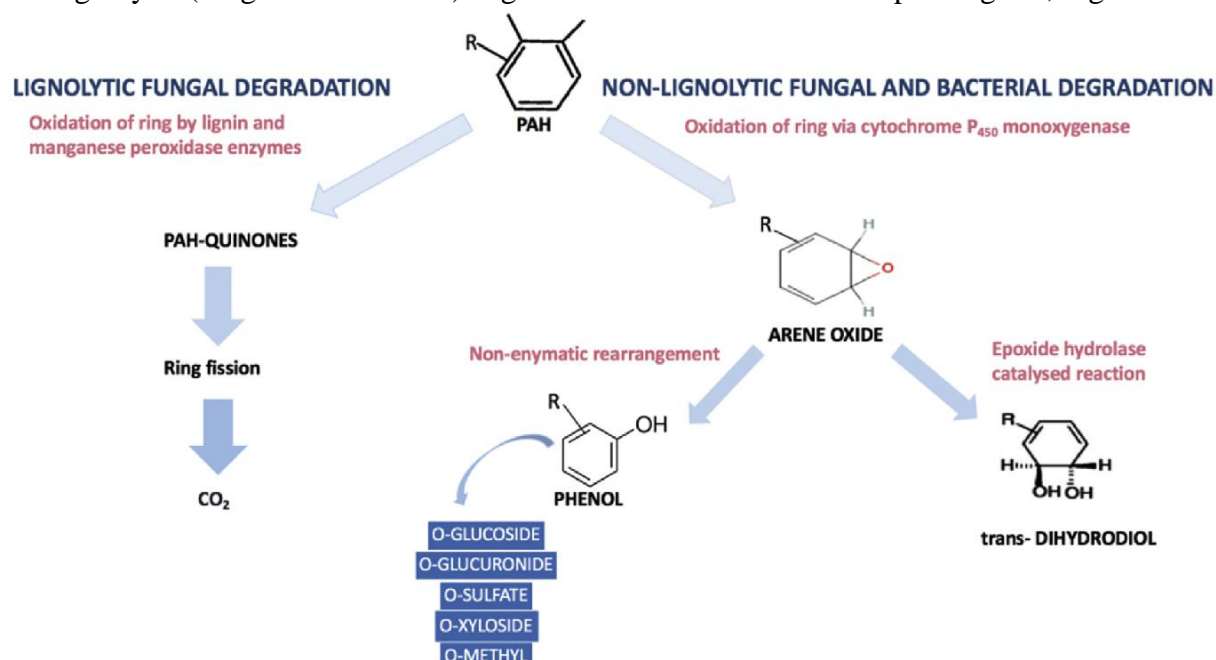


Fig. 18 – three main pathways of PAH degradation (Aydin et al. 2017)

Field et al. (1992) studied a number of strains of white rot fungi, to test their ability to remove PAHs and to register intermediate compounds originated in the process of degradation. Three genera – *Phanerochaete chrysosporium*, *Trametes versicolor* and *Bjerkandera adusta* were successful at degrading anthracene (99.2 % removal) and benzo(a)pyrene (83.1 % removal), in a period of 28 days. However, half of the strains produced a big amount of persistent metabolite anthraquinone, during the degradation of anthracene. Smaller amount of metabolites or none at all were produced out of benzo(a)pyrene. Metabolites of PAH degradation are not to be underestimated, they are often also toxic and often big amounts are being produced. For example oxygenated PAHs, which have similar origin like PAHs, but also appear as metabolites (Lundstedt et al. 2019).

Pozdnyakova et al. (2018) measured enzymes secreted by *Pleurotus ostreatus* and *Agaricus bisporus* in the presence of 3-ring PAHs, where *P. ostreatus* produced both laccase and peroxidase, which is more favorable for good results of degradation, concerning the final metabolites. They found out that when only laccase is present, the metabolites are rather accumulated than further degraded. Further degradation happens if both laccase and peroxidase are present, making *P. ostreatus* more suitable for fungal remediation. A different study by Pozdnyakova et al. (2016), focused on degradation and metabolites of fluorene and fluoranthene, again using *Pleurotus ostreatus*. The results showed a similar scheme, where laccase and peroxidase subsequently helped to oxidize entering pollutants as well as their metabolites.

Acevedo-Sandoval et al. (2018) made a success at degrading 10 different PAHs in an experiment with *Pleurotus ostreatus* and *Ochrobactrum intermedium*. When mixed in the contaminated soil together, some PAHs were removed completely: fluoroanthene (50 days), indene[1,2,3-cd]pyrene (80 days) and benzo[g,h,i]perylene (50 days). Other PAHs in the mixture were almost removed after longer period of time, 110 days, with following efficiency: anthracene by 96 %, pyrene by 86 %, chrysene by 98 % and benzo[a]anthracene by 98 %. The experiment included versions with both organisms separated, but the results showed they work better in a consortium.

Another study of *Pleurotus ostreatus* aimed at comparing degradation ability of fungi with naturally present bacterial colonies in the soil. Not only that the fungi was significantly better at removing pollutants, it also supported bacterial growth, further helping with remediation. Removal rates of this experiment also showed that PAHs with lower number of rings get degraded more easily. 86-96 % of 2-ring PAHs were removed, 63-72 % of 3-ring PAHs, 32-49 % of 4-ring PAHs and 31-38 % of 5 and 6-ring PAHs (Byss et al. 2008).

An aged soil contaminated with creosote often resists remediation by plants or bacteria due to unfavorable living conditions for these organisms, however a study by Eggen & Majcherczyk (1997) showed that for these cases using white rot fungi can be a good solution. In their experiment with *Pleurotus ostreatus*, aged benzo(a)pyrene originally present in the soil was degraded by 28 % in first month, artificially added benzo(a)pyrene even more – 40 % got degraded in first month. In next months the removal of the pollutant has decreased and after 3 months, 1 % of pollutant for mineralised to CO₂, which is ten times more than in a soil with no *P. Ostreatus*.

García-Delgado et al. (2014) compared a number of different strategies of bioremediation of creosote-contaminated soil, like natural bacterial degradation, wheat straw biostimulation or mycoremediation with *Pleurotus ostreatus*. The mycoremediation had the best results in all aspects of the experiment. These aspects were microbial development, eco-toxicity of soil and legal limits of pollutant levels. Only mycoremediation of *Pleurotus ostreatus* achieved to lower PAH levels below requirements of Spanish legislation for contaminated soils.

These results might lead to general conclusions that white-rot-fungi are excellent tool to remove PAHs, however some studies suggest that when pollution level is too high, the fungi remains in an inactive state, while bacterial colonies can keep growing. Also, the soil only inhabited by fungi with no or low microbial content does not create favourable environment for fungi to operate effectively (Canet et al. 2000). In an experiment of Winqvist et al. (2013), degradation by fungi in laboratory and field conditions are compared. They focused on heavily

contaminated soils and the presence of bacteria in-situ has shown to have a positive effect on overall degradation. Results however showed that very different processes occurred in the two experiments. In laboratory conditions, most of the degradation was done by fungi, whereas in the field, bacteria were much more active. More work in field scale still needs to be presented, because the differences between laboratory and in-situ experiments are often nonnegligible (Winqvist et al. 2013).

4 Material and methods

4.1 Chemicals

A mixture of PAHs was purchased as a standard of 16 US EPA priority PAHs, which list as acenaphthylene (ACY), acenaphthene (ACE), anthracene (ANT), benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[g,h,i]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (DBA), fluorene (FLU), fluoranthene (FLUO), indeno[1,2,3-c,d]pyrene (IPY), naphthalene (NAP), phenanthrene (PHE), and pyrene (PYR) in a 2000 mg/L mixture solution, from Chromservis, Czech Republic.

Other chemicals used were acetone, dichloromethane and *n*-hexane, each GC/MS grade, purchased from Chromservis, Czech Republic.

Before use, all glass-ware was cleaned by distilled water, followed by acetone and hexane, then dried in the oven at 150 °C, for 2 hours.

4.2 Characteristics of soil and wood chip substrate

The soil was brought from a site near Humpolec, Czech Republic, at long term trial site (49°33'16"N, 15°21'2"E), with altitude of 525 m a.s.l. and Cambisol soil type (Černý et al. 2010). More samples were collected from the site and then mixed together. The soil was then homogenised and sieved through a 5 mm gauze. The texture of the soil was sandy-loam of following composition: clay, 5.8 %, silt, 43.6%, sand, 50.6 %. The measured pH was 5.2. The soil already contained certain amount of PAHs, although they were below the quantifiable limits, ranging between 1.8 and 5.6 µg/kg dw, for individual PAH compounds.

Wood chips, which were later mixed with the soil to make a suitable substrate for lignolytic fungi, were obtained from the Crop Research Institute in Prague, Czech Republic. The lignocellulosic substrate came from waste apple tree branches (S1, 10-30 mm chips) and waste apple tree trunks (S2 30-50 mm chips). These were then mixed together in ratio 1:1 W/W (S3). Before use, each substrate was sterilised.

4.3 Plant seeds, fungal inoculum

A plant used for this experiment was maize (*Zea mays* L. Var. Colisee), purchased from KWS (Germany). The seeds were disinfected before sowing.

The fungal inoculum was prepared according to Garcia-Delgado et al. (2015). The *Pleurotus ostreatus* was grown on agar for two weeks, then moved to wheat grain. Before, the grain was half-cooked, drained, supplemented with 5 % of gypsum and filled in 1 L bottles. These were sterilised in autoclave in 151 °C for 2 hours. After inoculation, *P. ostreatus* was cultivated for 14 days. Wood chips were then inoculated with fungi-colonized grain and left to grow for 4 weeks, until ready to be mixed with the soil. The properties of this substrate were: drymatter, 39.3 % (W/W); pH (H₂O), 7.8; C_{tot}, 449 g/kg dw; N_{tot}, 12.0 g/kg dw; C/N, 44.1.

4.4 Experimental model

4.4.1 Establishment of the experiment

The experiment was carried out in outdoor, roofed conditions and lasted for 120 days.

Precipitation was controlled, light and temperature were natural. 1kg of soil was put in each of 32 plastic pots (h = 20.5 cm, d_{top} = 21.0 cm, d_{bottom} = 18.0 cm). These pots were divided by eight treatments in four replications. Treatments were prepared in different ways to simulate various approaches to PAH degradation in soil. Every one of them was spiked with a synthetic mixture of 16 individual US EPA priority PAHs (SV Mix 5, Restek, USA). The soil was spread thinly and the PAHs dissolved in acetone were added to the soil using a pipette at a rate of 100 ml of solution per kg of soil. Acetone was used as the carrier solvent as it solubilises the PAHs and is easily evaporated. (Smith et al. 2004) Spiking added 100 µg/kg dw content of each PAH species into the soil – total starting values of PAHs are listed in Table 1. After spiking, each pot was fertilised with NPK fertiliser - 100 mg N (NH₄NO₃ water solution), 32 mg P and 80 mg K (K₂HPO₄ water solution) per kg of soil. Description of each variation follows. 1. Natural degradation of PAHs in bare soil, no substrate of plants 2. Mycoremediation of PAHs with contribution of 10-30mm wood chips inoculated with *P. Ostreatus*, 3. Mycoremediation of PAHs by *P. Ostreatus* same as previous, but with bigger wood chips (30-50 mm), 4. Mycoremediation of PAHs by combination of previous two – 10-50mm wood chips with *P. Ostreatus* culture, 5. Phytoremediation of PAHs by using maize to grow in a contaminated pot, no added *P. Ostreatus*, 6. Combination of phytoremediation and mycoremediation, growing maize with 10-30mm inoculated wood chips, 7. Same as previous variation, but 30-50mm wood chips, 8. Combination of previous two variations, 10-50mm inoculated wood chips.

Maize was planted 8 seeds per pot, about 3 cm deep and watered with 500ml of demineralised water per pot.

4.4.2 Mid-experiment conditions and processes

After 15 days of germinations, plants were thinned to three to have similar size. Every week, pots were randomly moved to a different place to eliminate possible differences in site conditions. The pots were regularly weighted to maintain the moisture, which was kept at 60-70 % by adding demineralised water, when needed. All weeds were regularly removed, however fruiting bodies of *P. Ostreatus* which also appeared were left to grow.

After 120 days, maize and *P. Ostreatus* biomass were harvested and divided into roots and shoots. Corn cobs were not separated and measured separately even though originally planned to, because the yield was really small. Maize roots were washed to remove remaining soil. All three root parts and shoots of each pot were then weighted, sliced to smaller pieces, then homogenized and oven dried at 35 °C for 72 hours. Then milled to a fine powder. Soil samples were taken as a soil profile column. Three columns were collected out of every pot by a stainless steel tool, then mixed to form a sample, freeze-dried, ground with a mortar and sieved through a 2 mm sieve. Then they were stored at -20 °C in Petri dishes covered in foil before analysed.

4.4.3 Laboratory analysis of PAH content

PAHs were measured for maize roots, maize shoots, fungal biomass and for soil. Soil was also measured at start of the experiment, to know the exact difference in values after 120 days. Firstly 5 g of the sample was weighed into a flask (100 mL), then added 30 mL of hexane-acetone mixture (1:1, V/V) and put into ultrasonic extractor for 30 min. The mixture was then filtered and rinsed with 5 ml of hexane. This was done two more times, before evaporated in a rotary evaporator, at 40 °C to near dryness. This was then dissolved in 5 mL of hexane and concentrated for 1-2 mL. After elution of cartridges, samples were tested. For roots, shoots and

fungal biomass, same method as for soil was used. The samples weighted 5, 5 and 2 g respectively. For the measurement, gas chromatograph was used. A five-point calibration curve was given, for each PAH compound. Detailed information about the analysis was given by Košnář et al. 2018.

Results were calculated by finding average value among four repetitions of a variety, then calculating the difference between initial and 120-day values, transferred to percentage of how much PAHs got degraded. Results for soil, maize roots, maize shoots and *P. Ostreatus* biomass were treated separately. Software used for these calculations and creation of charts with presented results was Microsoft Excel 2010 (Microsoft Corporation, 2010) and Google Sheets: Free Online Spreadsheets for Personal Use (Google Commerce Ltd, 2020).

5 Results

5.1 PAHs in plant and fungal biomass

The content of PAHs in maize biomass was overall low, but showed much higher values in samples with *P. Ostreatus* inoculated substrate. As described before, four variations in four repetitions were prepared with maize no substrate in the soil, *P. Ostreatus* inoculated substrate with 10-30mm wood chips (S1), 30-50mm wood chips (S2) and the combination of the two – 10-50mm wood chips (S3). The average value for each of four variations was calculated. Low molecular weight PAHs (LMW PAHs) and some of medium molecular weight PAHs (MMW PAHs) were the only compounds that returned mentionable values. High molecular weight PAHs (HMW PAHs) showed too low to no content at all, therefore are not mentioned in Figure 19. Roots were the only biomass that had mentionable content of PAHs, maize shoots and fungal biomass had little to no measurable content of PAHs, therefore these are also not listed in the results. Naphthalene, being the most simple measured substance, showed highest content. In S3 variety, the amount of naphthalene in roots was 13,7 $\mu\text{g}/\text{kg}$, while with no substrate, the contents remained as low as for other measured substances. The difference between values in non-substrate and substrate variations could be caused by activity of fungal enzymes, which increase bioavailability of PAHs, which are then more easily uptaken by plant roots.

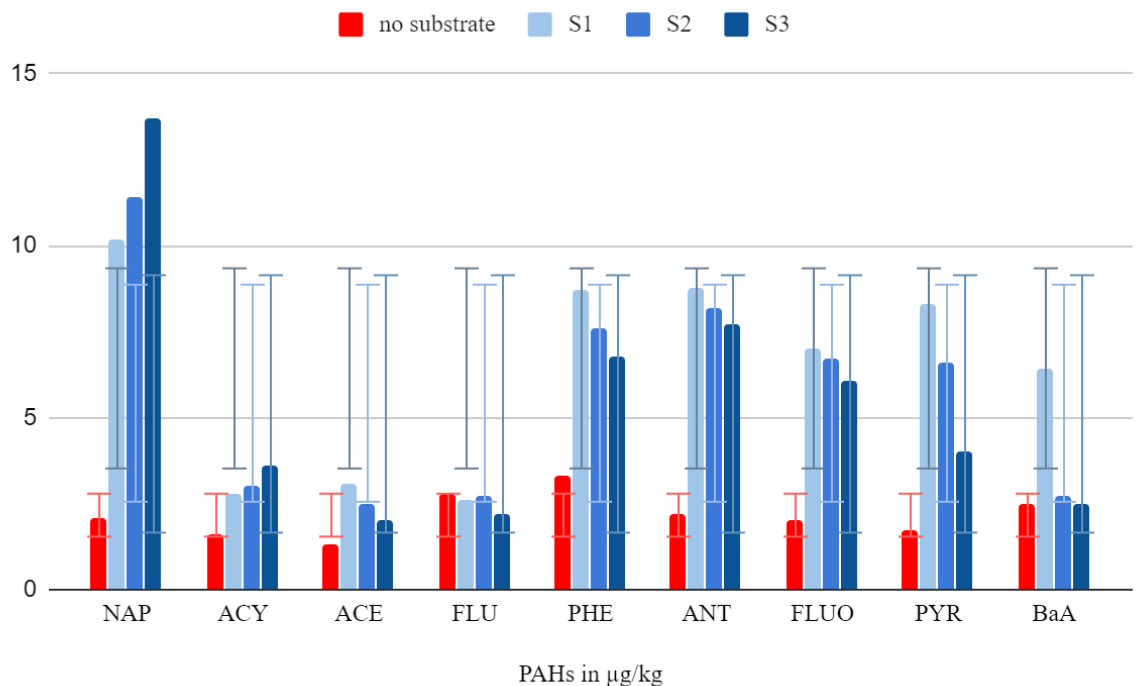


Fig. 19 – Contents of PAHs in maize roots biomass in $\mu\text{g}/\text{kg}$ at the end of the 120-day experiment. Only LMW PAHs and MMW PAHs are listed, as these were the only ones with measurable values (NAP – naphthalene; ACY – acenaphthylene; ACE – acenaphthene; FLU – fluorene; PHE – phenanthrene; ANT – anthracene; FLUO – fluoranthene; PYR – pyrene; BaA – benzo(a)anthracene). For each PAH, 4 varieties are compared – no substrate in the soil; *P.*

Ostreatus inoculated substrate with 10-30mm wood chips (S1); 30-50mm wood chips (S2); the combination of these two – 10-50mm wood chips (S3).

5.2 PAH degradation in soil samples

All soil samples had their PAH content measured at day 0, when the experiment started. At the end of the 120-day experiment, the percentage of degradation in comparison with original value was calculated using this formula:

$$d \% = (C_0 - C_{120}) / C_0 * 100$$

C_0 stands for initial content of PAHs, C_{120} is the terminal value and d shows how much degradation took place. This number is demonstrated as a percentage of original PAH content for which it got degraded. Results of all 16 PAHs were divided into groups of LMW PAHs, MMW PAHs and HMW PAHs and are listed in Figure 20, Figure 21 and Figure 22.

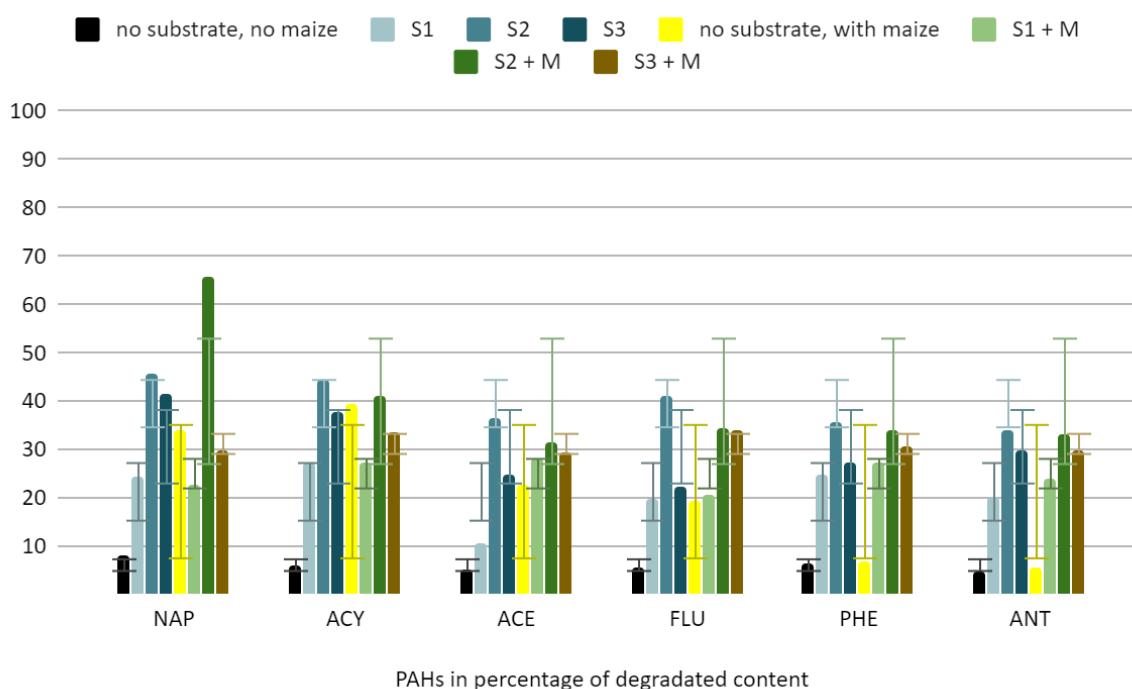


Fig. 20 – Degradation of LMW PAHs in soil at the end of 120-day experiment. Values are shown in percentages of degradation for all eight variations. Low molecular weight PAHs in this graph are naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE) and anthracene (ANT).

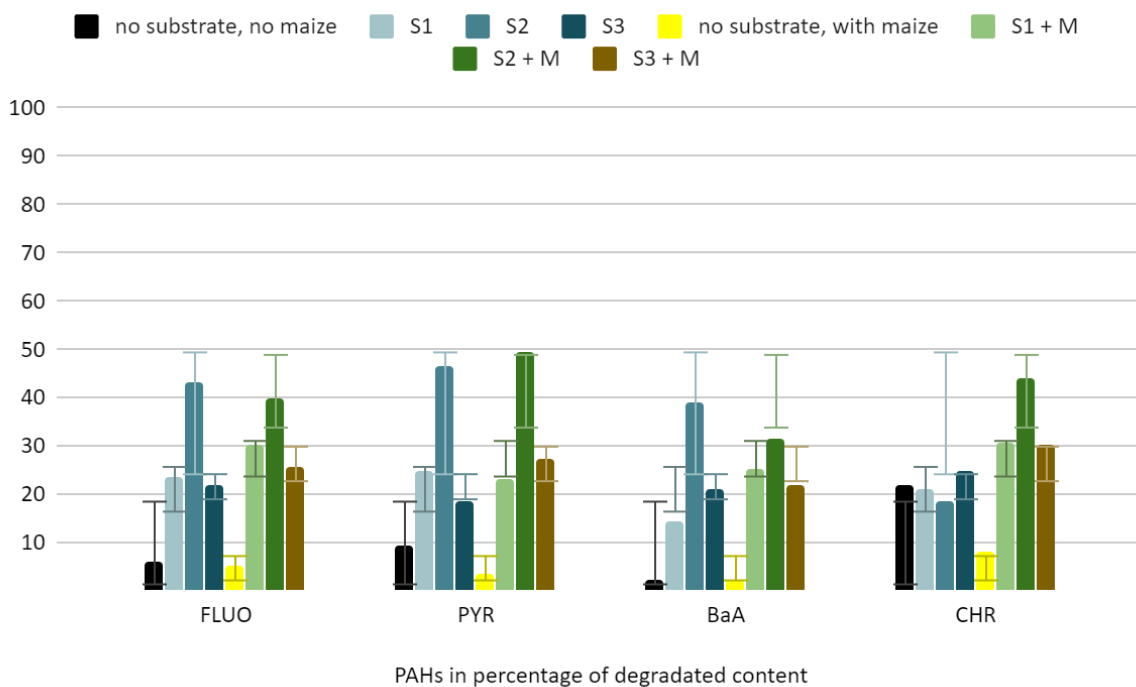


Fig. 21 - Degradation of MMW PAHs in soil at the end of 120-day experiment. Values are shown in percentages of degradation for all eight variations. Medium molecular weight PAHs in this graph are fluoranthene (FLUO), pyrene (PYR), benzo(a)anthracene (BaA) and chrysene (CHR).

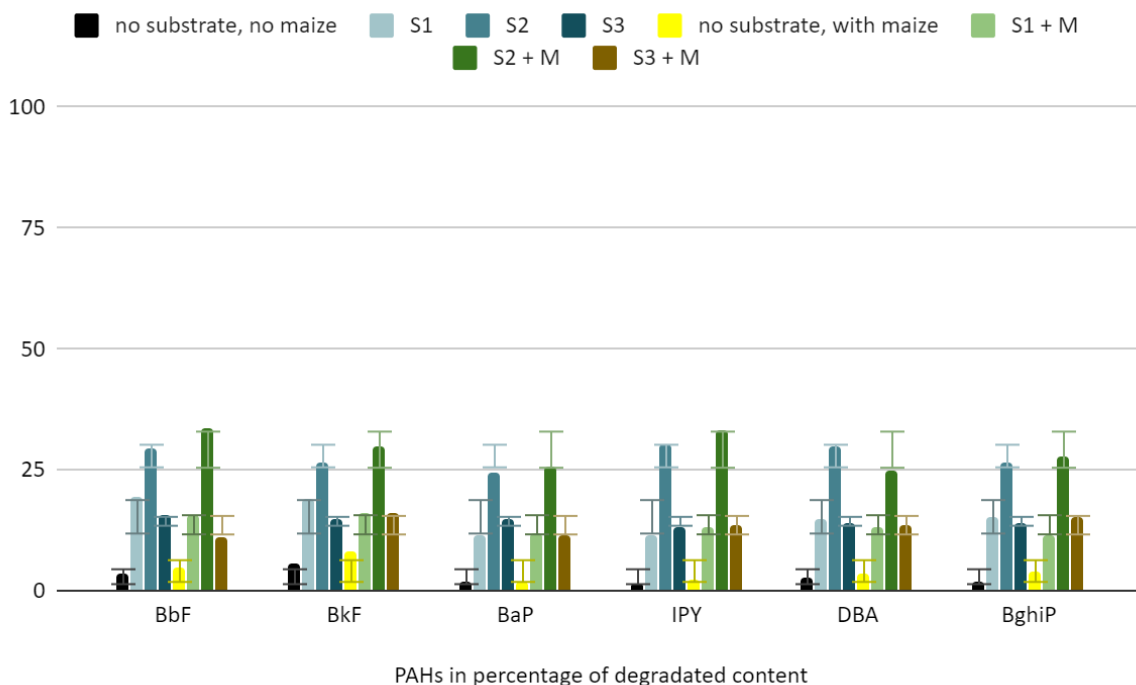


Fig. 22 - Degradation of HMW PAHs in soil at the end of 120-day experiment. Values are shown in percentages of degradation for all eight variations. High molecular weight PAHs in this graph are benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IPY), dibenz(a,h)anthracene (DBA) and benzo(g,h,i)perylene (BghiP).

The differences between non-assisted and assisted remediation (either with inoculated substrate, plants, or both) were significant. For LMW and MMW PAHs, non-assisted remediation did not exceed 8,8 µg/kg, which is less than 10 % of the pollutant content at day 1, for HMW PAHs, the degraded amount was even smaller, for example benzo(k)fluoranthene degradation only reached 5,7 µg/kg and for indeno(1,2,3-cd)pyrene it was only 1,5 µg/kg.

5.2.1 Fungal inoculate-assisted remediation

The addition of fungal-inoculated substrate proved to have an effect on degradation of PAHs. Among 3 variations of added substrate, S2 (30-50mm wood chips) showed to be the most effective. As seen from Figure 20, 21 and 22, this type of substrate exceeded other types in all cases, except for chrysene, where all three substrates showed very similar results. The average amount of degradation for LMW PAHs was around 40 % and 43 % for MMW PAHs, if we do not include chrysene, which got remediated for 18,4 %. HMW PAHs, which have naturally the highest resistance got degraded by 28 % on average, in variation S2. In S2 category, the original content of total PAHs was about 1500 µg/kg dw. After degradation, the content dropped to 955 µg/kg dw. This rate of degradation lowered contents of PAH below the maximum limit of PAHs (1000 µg/kg dw) according to the Public Notice No. 153/2016 for agricultural soils in the Czech Republic.

Variations S1 and S3 (10-30mm and 10-50mm respectively) had overall lower effectivity, although still showed some activity in comparison with no substrate used. For LMW PAHs, S3 was more effective than S1, with average degree of degradation of 30,5 %, while S1 reached only 21,3 %. For MMW and HMW PAHs, S1 and S3 had generally very similar results. MMW PAHs showed on average 21 % level of degradation, while HMW PAHs only 14-15 %.

Amount of fungal biomass grown on surface of the soil was measured, but did not significantly correlate with the results presented above, as the amount of biomass was overall small and did not show any visible trend. Weighted amount of *P. Ostreatus* biomass was 1,7-2,6 g/pot dry weight. Even though the amount does not show any substrate preference, it proves fungal activity in the soil and its high colonization with *P. Ostreatus*.

5.2.2 Phytoremediation

Growing maize in contaminated soil alone, with no substrate, only showed results for LMW PAHs, with the exception of phenanthrene and anthracene, where the effectivity was very low (6,7 % and 5,5 % respectively). For naphthalene, acenaphthylene, acenaphthene and fluorene, the results were nonnegligible (NAP 33,9 %, ACY 39,3 %, ACE 22,8 %, ACE 19,6 %). For the rest of PAHs, the average degraded amount was only 4 % on average.

Presence of pollutants did not have any noticeable effect on maize vitality – measuring of plant height during the experiment did not show any differences between the four variations.

6 Discussion

The articles on remediation of PAHs and their effects on human and animals change in time. In the newest articles published by Agoun-Bahar et al. (2019), in some cases opinions on toxicity of single PAHs have changed, but these are still under close focus. The problem with negative effects of these pollutants is their highly variable contraindication – depending on the environment, amount and especially composition of PAHs present, different effects take place. Some of the 16 USEPA PAHs have been confirmed to be human carcinogens, other are only possible carcinogens (Andersson & Achten 2015). The fact that they naturally always occur together however implies, that even though some may be less toxic than others, it is still highly advisable to focus on their remediation.

Bioremediation has shown to be very effective and its advantages are nonnegligable. Some authors, for example Feizi et al. (2019) focused on bacterial degradation, but in our experiment bacteria only had a small effect, even lower than expected (in variation without substrate nor plants). This shows bacterial degradation might be effective in some cases, but in field scale it could be too slow and ineffective. Fungal remediation is more easy to establish and control, with possibly faster results.

A research by Treu & Falandysz (2017), which mentioned funghi being able to solve obstacles in the soil, corresponded with our experiment, where *P. ostreatus* was able to produce fruiting bodies in drought cracks and by the sides of the pots, even though it usually doesn't grow directly on soil (fruiting bodies of *P. ostreatus* are captured on picture attachment). This fact might imply that funghi could continue growing and remediating soil long after it has been incorporated into the soil.

6.1 Uptake of PAHs by maize

One of the biggest concerns about growing crops on contaminated soil was whether they will uptake and accumulate PAHs. Research articles by Kacálková & Tlustoš (2010) and Onyedikachi et al. (2019) predicted the PAH uptake would be in low amounts and the results of this work showed similar trend, which is that roots are able to uptake PAHs to some extent, but these do not travel further into plant. Even though some species were found to accumulate pollutants in above-ground biomass (Chen et al. 2019), *Zea mays* did not and its levels stayed below permitted limits. This means the biomass can be safely used for further processing, for example for animal feed. Also, the vitality of single plants was not affected, as they all had overall similar height which further supports this claim.

As expected, when combined with fungal inoculated substrate, maize absorbed more pollutants, caused by fungal enzymes, making PAHs more bioavailable, as previously described by Haritash & Kaushik (2009).

6.2 Contents of PAH in soil

Not only plant biomass but also soil presents possible source of devaluation of livestock products. As suggested by Jurnanz et al. (2012), it is important that in grazing sites the soil does not exceed acceptable limits of PAH content. After only 120 days, we were able to drop PAH levels under this limit, meaning the remediated soil would already be suitable for fodder crops or even crops intended for direct consumption. This supports suggestions of previous research, for example by Sakshi et al. (2019) to more commonly incorporate biodegradation techniques in field conditions.

It was expected that when combining plants and fungal substrate, degradation rates would be noticeably higher. Symbiosis between plant roots and fungal enzymes was supposed to remediate pollutants more easily. However, this was not confirmed, as the differences between variations with and without plants were small and did not show any trend which could be caused by the antagonists effect of maize and fungal substrate when combined together. Nevertheless, using this treatment the PAH removal in soil enabled a highest PAH removal in soil. Therefore, mycoremediation assisted by plants could be seen as a promising bioremediation treatment in PAH contaminated soil.

7 Conclusion

- Characteristics of polycyclic aromatic hydrocarbons have been summarised, with the accent on their toxic effects, which were proven to be carcinogenicity, teratogenicity and ability to cause various respiratory issues. Presence of these pollutants in the environment causes concern and efforts to remove them have been made. Even though PAHs are not easily uptaken by plants, cattle and other farm animals are exposed to them by ingesting soil, which is sometimes highly polluted and causes further contamination of livestock products.
- Information gathered in the literature suggested that using white-rot fungi for remediation of PAHs would be effective. This was confirmed. Comparison between variations with and without fungal substrate showed high effectivity of fungal ligninolytic enzymes, which lowered PAH content below the maximum limit of PAHs (1000 µg/kg dw) according to the Public Notice No. 153/2016 for agricultural soils in the Czech Republic.
- PAHs did not accumulate in planted maize and also did not affect its vitality, as the size and weight of single plants were very similar. This fact contributed to the conclusion that it is safe to grow fodder or other crops on contaminated soil, as they do not get affected and therefore present a low risk of causing damage in further foodchain. This however only applies to crops with above-ground usable biomass, as roots absorbed some of the pollutants.
- Hypothesis about effective cooperation between plants growing in polluted soil and fungal inoculated substrate was not confirmed, as the amount of degraded PAHs was not significantly higher than in variations without plants. This could be caused by plant species or premature termination of the experiment, as some enzymatic activity continues even post-harvest. More research needs to be done on this topic.
- Overall, the speed and effectivity of bioremediation has shown to be highly positive and adds to previous suggestions to use this type of PAH degradation in field scale.

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