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Research Article

Selective screening: isolation of fungal strains from contaminated soils in Austria

Selektives Screening: Isolierung von Pilzstämmen aus schadstoffbelasteten Böden in Österreich

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Summary

Microorganisms are potent contributors to maintaining a safe environment as they are able to degrade organic toxicants. For environmental applications, mostly bacteria are used while fungal strains have received less attention. However, they are able to degrade highly persistent organic contaminants and survive extreme conditions, and may thus be promising organisms. To find new fungal candidates for these applications, twelve soil samples from polycyclic aromatic hydrocarbon (PAH) contaminated sites in Austria were used to isolate fungal strains. A microplate screening method using PAH contaminated soil as inoculant was set up to isolate fungal strains being able to live in presence of toluene, hexadecane, or polychlorinated biphenyl 126. Not many microbial strains are known that degrade these three contaminants, while the PAH contamination acted as selective pressure for the soil microbiota. After obtaining pure cultures, the fungal strains were further screened for their ability to live in the presence of one of the three contaminant substrates. The potential for technical application of the 11 best performing strains, identified using ITS and 18S rDNA, is discussed. The presented microtiter plate screening method is a cost efficient and quick approach to identify fungal strains for pollutant degradation and results in candidates with a high relevance for bioremediation techniques.

Keywords: biodegradation, bioremediation, fungi, screening, contaminated soil

Zusammenfassung

Mikroorganismen leisten durch ihrer Fähigkeit organische Schadstoffe abzubauen einen wichtigen Beitrag zur Erhaltung einer saubere Umwelt. Für Anwendungen in der Umwelt finden meist Bakterienstämme ihre Anwendung, während viele Pilzstämme auch die nötigen Eigenschaften besitzen, persistente, organische Schadstoffe abzubauen. Sie haben zusätzlich den Vorteil, extreme Bedingungen zu überleben. Pilze eignen sich als vielversprechende Kandidaten für biologische Sanierungen. Um mögliche Kandidaten zu finden, wurden in unserer Studie zwölf mit polyaromatischen Kohlenwasserstoffen (PAKs) kontaminierte Bodenproben von verschiedenen Standorten in Österreich als Inokulum zur Isolierung von Pilzstämmen genutzt. Hierfür wurde ein selektives Mikrotiterplatten Screening in Gegenwart von Toluol, Hexadekan oder polychloriertes Biphenyl (PCB) 126 entwickelt. Die drei Schadstoffe wurden gewählt, da nicht viele Mikroorganismen diese abbauen können, während die PAKs als selektiver Druck für die Bodenmikroorganismen wirkte. Von den resultierenden Pilzkulturen wurden Reinkulturen erstellt und anschließend ein weiteres Mal auf ihr Wachstum mit den drei Schadstoffen als Kohlenstoffquelle getestet. Das technische Anwendungspotential der elf Stämme mit dem besten Wachstum, die mit Hilfe von ITS und 18S rDNA Sequenzierung identifiziert wurden, wird diskutiert. Die präsentierte Screening Methode ist eine effiziente und schnelle Methode, um abbauende Pilzstämme mit einem hohen Anwendungspotenzial zu identifizieren. Durch die Isolierung aus österreichischem Boden eignen sich die isolierten Pilze besonders für eine Anwendung im Schadstoffabbau im zentraleuropäischen Raum.

Schlagworte: biologischer Abbau, biologische Sanierung, Pilze, Screening, kontaminierte Böden

1. Introduction

Environmental pollution with man-made contaminants poses potential risks for humans, animals and plants due to their persistence and toxicity. Contaminants are very different in their chemical structure, show different abundance and stability in the environment and differ in the accessibility for microorganisms. In addition, they can strongly adsorb to soil particles and remain immobilized (Boonchan et al., 2000; Pinto and Moore, 2000). Contaminants have a large extent: from crude oil ingredients as benzene, toluene, ethylbenzene and xylene (BTEX), to polycyclic aromatic hydrocarbons (PAHs) from coal and tar residues, hexadecane from diesel to chemical liquid waste as polychlorinated biphenyls (PCB). The removal of these toxic compounds is a challenge and can be very cost intensive. New technologies and cleanup methods together with a better understanding of used methods, to improve degradation time and rate, are needed.

Biofilter systems are a common gas or wastewater treatment technologies that use biological mechanisms to degrade contaminants. They are cost-effective compared to physicochemical processes, especially in case of high gas flow rates with low concentrations of volatile organic compounds (Maestre et al., 2007). Mostly bacteria are used in these systems, but fungal strains can also be good candidates. Fungi have the advantage of being able to withstand harsh conditions like low water availability, varying pH values or changes in temperature and are able to degrade great variety of contaminants (Cox, 1995). Additionally, bacterial gas biofilters have poor degrading abilities concerning hydrophobic contaminants, as they are poorly absorbed due to their low water solubility (van Groenestijn et al., 2001). One technical option to overcome this problem is fungal strains grown on inert packaging material (van Groenestijn et al., 2001). Examples of successful usage are *Exophiala jeanselmei*, used to treat styrene polluted air (Cox, 1995), and *Aspergillus niger,* used for degradation of hexane (Spigno et al., 2003; Groenestijn and Kraakman, 2005).

Another option to use fungal strains is residual contaminations in soils, which often remain untreated after the excavation of highly contaminated hot spots due to the high costs. In these situations, *in situ* bioaugmentation may be a good alternative. Although it requires longer time compared to traditional approaches, the bioremediation approach may be preferable due to its lower cost and environmental impact (Harms et al., 2011). Bioaugmentation might be a promising approach depending on the microbiota and their survival rate in the soil (Mrozik et al., 2010). Therefore, strains from contaminated soil might be a solution, as they are already adapted to the contaminant rich environment. Bacteria and fungi are ubiquitously present in sediments and play a significant role in natural biodegradation (Atlas, 1995). Fungal strains have extensive metabolic capabilities eligible for decontamination, but their potential use in bioremediation to date has only received limited attention (Harms et al., 2011). Due to the fungal ability to grow in a hyphal form, they can reach and penetrate contaminated soil easily (April et al., 1999). There are limitations in fungal application, which have to be kept in mind, for example, fungal degradation can be a slower process, the strains might need longer time to adapt and sometimes degradation leads to intermediates that were not completely removed by the strain (Sasec and Cajthaml, 2014; Kulshreshtha et al., 2014; Zahid Maqbool et al., 2016). Interaction with bacteria in degradation processes has not been studied extensively, but might be very important for the improvement of remediation performance.

The group of white-rot basidiomycete fungi and their molecular degradation tools, that is, extracellular ligninolytic enzymes and accessory enzyme, are normally used for lignin degradation and has been found to be useful for contaminant degradation. However, white-rot fungi typically grow in compact wood and favor acidic condition (Marco-Urrea et al., 2015). Therefore, known non-ligninolytic fungal strains with the possibility to live on contaminants are often better candidates for bioaugmentation, as they are able to live in extreme conditions such as high or changing pH or desiccation.

To identify key players of fungal degradation in contaminated soil, for the first time we set up a microtiter plate screening method based on the screening method described by Blasi et al. (2016) and developed a quick and high throughput screening method using the contaminated soil as inoculant. Twelve soil samples were used to obtain a broad range of fungal strains and investigate differences between the sites and varying PAH contamination that acted as selective pressure for the microbiota. We were able to isolate 93 different fungal strains from the first screening where contaminated soil was used as inoculant and growth in the presence of toluene, PCB 126 and hexadecane was tested. These contaminants were chosen as they offer a broad range of different chemical structures and not many microbes are known to be able to degrade PCB. Pure fungal strains were cultivated and growth in the presence of the three contaminants was confirmed re-

peating the microtiter plate screening method. The best growing 11 strains were identified by ITS and partial 18S rDNA sequencing data. These strains are discussed for their potential application in the field.

2. Materials and Methods

2.1 PAH Analysis of contaminated soil samples

Soil samples from different contaminated sites were provided by ESW Consulting Wruss ZT GmbH. The samples were dried with Na_2SO_4 (Merck Millipore) and 30 g of homogenized soil sample was extracted on a Soxhlet apparatus for at least 6 hours using 150 mL of cyclohexane (LGC standards GmbH) as a solvent, containing six deuterated internal standards (*naphthalene D8, acenaphthene D10, phenanthrene D10, fluoranthene D10, benzo[a]anthracene D12, and benzo[a] pyrene D12*, Dr. Ehrenstorfer GmbH). After extraction, the solution was brought to 150 mL (to compensate for possible solvent loss during extraction). 300 µl of this extract was transferred into a cyclohexane preconditioned clean up column filled with aluminum oxide (Merck production chemicals, 1.5 mL) and Na_2SO_4 and then flushed twice with 600 µl of cyclohexane. The resulting extracts were then analyzed by large volume – gas chromatography – mass spectrometry (Agilent 7890A coupled to a 5975C inert XL mass spectrometer) using a ZB-5MSi fused silica capillary column (5%-diphenyl- 95%-dimethyl polysiloxane, 30 m × 0.25 mm ID × 0.25 µm) from Phenomenex.

2.2 Microtiter plate screening method for pollutant degrading fungal strains

2.2.1 Screening method for pollutant degrading fungi using PAH contaminated soils as inoculants, isolation and cultivation of pure cultures

Unsieved soil of each sample (approximately 0.15 g) was dissolved in 1 mL of 0.9% sterile NaCl solution and vortexed to gain a homogenized liquid inoculant. 20 µl of each inoculant were used for the microtiter plate screening method using a 96 wells plate. Three different contaminants were used to identify new fungal species being able to grow in the presence of PCB 126 (10 ng μl^{-1} PCP 126 in isooctane, Dr. Ehrenstorfer GmbH), hexadecane (99% analytical grade, Alfa Aesar) or toluene (Merck KGaA, Darmstadt, DE). Per contaminant 150 µl of media, 50 µl of contaminant solution and 20 µl of the inoculum inoculant were used for hexadecane and PCB 126. Plates were kept at room temperature on a shaker. Toluene (5%) was diluted with dibutyl phthalate in a beaker in a glass vacuum desiccator and plates were stored next to it to expose them to the volatile phase. Toluene plates were filled with 200 µl of medium plus 20 µl of inoculant. All tests were performed in triplicates, a negative control (medium without inoculant) was included on each plate. In addition, a positive control was carried out for each soil: 200 µl of a glucose medium and 20 µl of inoculant to investigate the number of fungal colonies deriving from the contaminated soil samples with glucose as carbon source. Growth of fungal species was measured through changes in the optical density (OD) at 700 nm with a microtiter plate reader Infinite M 1000 (Tecan, CH, set at 24°C, 70.8 rpm, wavelength: 700 nm, bandwidth: 5 nm) and OD values were corrected with values of the negative control. After 10 days, fungal growth was observed in all wells, the screening method was stopped and the liquid of the microtiter plate wells was plated on 2% malt extract agar (MEA: 2% malt extract, 2% D-glucose, 0.1% bacto peptone, 2% agar) and Rose-Bengal Chloramphenicol agar (Merck) from the wells with the contaminant but also from the positive control to isolate fungal strains growing on glucose and PAHs. Fungal strains were isolated and pure cultures were cultivated.

2.2.2 Screening of pure cultures for growth on contaminant substrates toluene, hexadecane and PCB 126

In order to test the ability of each isolated strain to live in the presence of the contaminants and to identify the best growing strains, the microtiter plate screening was repeated using pure cultures as inoculant rather than PAH contaminated soil. For this experiment, 1 cm^2 of fungal biomass was diluted in 1 mL of 0.9% of sterile NaCl containing glass beads (Carl Roth, DE) and was homogenized on a Ribolyzer (FastPrep-24 Instrument, MP Biomedicals, CA) for 5 sec at 4 m s^{-1}. The screening protocol for PAH contaminated soils was also used to grow strains in triplicates at room temperature in the presence of hexadecane, PCB 126 or toluene. Additionally, positive and negative controls, as previously described, were set up and the plates were incubated for 27 days.

2.3 DNA extraction and sequencing

DNA extraction of the eleven best growing strains was done according to the protocol described in Sert and Sterflinger (Sert and Sterflinger, 2010). In this procedure, the fungal cells are disrupted through glass beads using a Ribolyzer for 20 sec at 4 m s^{-1} twice, in-between samples

were cooled on ice. DNA is recovered through Ethanol precipitation. The extracted DNA was tested on its purity on a Nanodrop 1000 spectrophotometer (Thermo Fisher) and bands were detected on an electrophoresis gel 1.5 % (w/v). PCR of the ITS or 18S rRNA sequences was done using the primer pair ITS1 / ITS4, further NL1 / NL4 and NS5 / NS8 at a concentration of 0.5 µM. For the PCR the protocol of the Phusion Polymerase (Thermo Fisher) was used, including 2 U μl^{-1} Phusion polymerase, Phusion HF buffer (Thermo Fisher) and 10 mM dNTPs. The Thermocycler (Biorad) was set up at 98°C (30 sec) for initial denaturation followed by 34 cycles at 98°C/20 sec, 60°C/30 sec and 72°C/105 sec and ended by the final elongation 72°C/120 sec. After checking the obtained PCR products on an electrophoresis gel 1.5% (w/v), the products were purified using the QIAquick PCR Purification Kit (Qiagen). Sequencing of the products was performed by Sanger Sequencing (Microsynth Austria). Sequencing results were compared with the online databases provided by the National Center for Biotechnology Information using the BLAST search program (Altschul et al., 1997) and sequences were deposited in the NCBI nucleotide database under the accession numbers listed in Table 1. Additionally, we did morphological and taxonomic analysis according to Domsch et al. (2008).

3. Results and discussion

3.1 PAH measurements

The total concentration of 16 US EPA PAH ranged from 20.53 mg kg^{-1} DW (soil sample (S) 10) to 867.77 mg kg^{-1} DW (S 6). S 1—6 contained a lower proportion of high molecular weight PAHs (HMW, ≥ 4 aromatic rings, Figure 3) compared to S 7—12. According to literature (Lladó et al., 2013), samples with a high proportion of HMW PAHs are most likely aged contaminations due to the quicker dissipation of low molecular weight (LMW) PAHs in the field.

3.2 Microtiter plate screening

We were able to successfully adapt the microtiter plate screening method described by Blasi et al. (2016) to isolate 93 fungal colonies using the contaminated soil as inoculant. The soil contaminated with PAHs, acted as selective pressure for the microbiota to be able to isolate the fungal strains adapted to the contaminated environment. Two different microtiter plate screenings were performed:
- with soil as inoculant followed by isolation of fungal colonies (3.2.1)
- with pure strains after isolation and cultivation from the soil (3.2.2)

3.2.1 Microtiter plate screening with soil inoculant

As described above, soil samples varied in PAH content (Figure 3 and Table 2). This had a direct effect on the number of colonies isolated from the soil (Figure 1a, b). In the first plate screening, mixed cultures were obtained and resulted in 93 pure cultures. The highest amount of PAH was measured in S6. Nevertheless we could isolate two fungal colonies, one of them resulting in a successful candidate of the screening. In 6 out of 12 samples, a link between the proportion of HMW to total PAH concentration and the total number of isolated fungal communities was indicated by a strong linear regression (Figure 1a). The proportion of HMW / total PAH can be linked to the age of the contamination (Lladó et al., 2013). Accordingly, microorganisms in soil samples with lower proportion of HMW PAHs tend to be less adapted to contaminant degradation compared to microorganisms that could adapt to the contamination over a longer period of time in soil samples with a higher proportion of HMW PAHs. The 6 samples excluded from the regression analysis (S 7-12) were samples of very high HMW proportion, indicating high age. In these samples the microbial composition may have further changed because most degradable contaminants were already degraded. No correlation between individual PAHs and the ability to survive one of the three target contaminants could be found.

3.2.2 Microtiter plate screening with pure cultures

After the cultivation of pure cultures, the microtiter plate screening was repeated. Results are represented in Table 3. All values were corrected with the values of the negative controls.

For the 93 isolated fungal colonies, survival was highest in the presence of hexadecane (76%) and toluene (56%), likely due to a lower toxicity compared to the chlorinated PCB (LD50 Rat oral: Toluene 2.6 to 7.5 g/kg, PCB 4250 mg/kg, Hexadecane not lethal; cf., U.S. National Library of Medicine). 20% of the colonies showed slow or no growth in the pure strain screening, which can be explained by mutualism or by an additional carbon source

such as dissolved organic carbon from the soil which was missing in the second screening using pure cultures.
Interactions and mutualism of different strains in the microbiota might play an important role for degradation as a study by Boonchan et al. (2000) showed.
After eliminating similar strains based on their macroscopic and microscopic appearance, we chose 11 fungal strains (Table 4) that showed a high increase in biomass (Absorbance ≥ 0.5 for hexadecane or toluene, increasing absorbance with PCB) and more than one microtiter plate setup (Figure 2a, b, c). These strains were chosen for identification through Sanger sequencing of the ITS or 18S rDNA. We used different primer pairs to get the optimal coverage and identity using NCBI BLAST (Table 1). For most of the strains, growth in the presence of PCB 126 was poorly visible. Only strains BL3 and U, identified as *Ochroconis longiforum* and *Pyrenochaeta inflorescentiae*, showed clear increase of OD values over 27 days. In the presence of hexadecane, all the strains but alpha 14 and BL4 showed high increase of OD values. The two fungal strains only increased their biomass at the end of the test, and therefore, might need longer growth time. For 9 strains, an increase of OD in the presence of toluene was visible.
Nine out of 11 strains (as given in Table 1) have previously been reported as isolated from the contaminated sites. Especially good results in the microtiter plate screening showed *Purpureocillium (Paecilomyces) lilacinum.* The strain showed the greatest increase of biomass in the presence of hexadecane and toluene. It was reported to be isolated from various contaminated sites: plastics contaminated soil (Pradeep et al., 2013), from the acidic river Rio Tinto (Oggerin et al., 2013) and — classified as *Penicillium lilacinum* — from mine drainage containing high amounts of cadmium (Tatsuyama et al., 1975). The strain is very stress resistant, survives extreme conditions concerning pH values and heavy metal concentration but is also medically relevant as opportunistic pathogen (Luangsa-Ard et al., 2011).

Table 1. Phylogenetic classification of the ITS/18S rDNA coding sequences of the fungal isolates
Tabelle 1. Phylogenetische Klassifikation der ITS/18S rDNA kodierenden Sequenzen der Pilzisolate

No	Primer Pair	Closest identified phylogenetic relatives [EMBL accession numbers]	Query cover	Ident	ACBR strain No	accession No
α 14	NL1/NL4	*Doratomyces purpureofuscus* (Cephalotrichum purpureofuscum) genomic DNA sequence contains 28S rRNA gene, strain UAMH 9209 [LN851018.1]	99%	99%	MA6020	KY454753
O	NS5/NS8	*Roussoella intermedia* strain CBS 170.96 18S ribosomal RNA gene, partial sequence [KF443390.1]	99%	100%	MA6025	KY454758
Y	NL1/NL4	*Purpureocillium sp.* JCM 28545 gene for 28S ribosomal RNA, partial sequence [LC134235.1]	100%	99%	MA6015	KY454754
BL 3	NL1/NL4	*Ochroconis longiphorum* strain CBS 435.76 28S ribosomal RNA gene, partial sequence [KF156135.1]	96%	99%	MA6021	KY454755
BL 4	NL1/NL4	*Ochroconis longiphorum* strain CBS 435.76 28S ribosomal RNA gene, partial sequence [KF156135.1]	99%	99%	MA6017	KY454756
V	ITS1/ITS4	*Penicillium janthinellum* [EF634422.1]	100%	99%	MA6016	KY454760
Z	ITS1/ITS4	*Pyrenochaeta inflorescentiae* [EU552153.1]	97%	98%	MA6019	KY454761
H	ITS1/ITS4	*Penicillium canescens* [AY373901.1]	100%	99%	MA6018	KY454762
U	NL1/NL4	*Pyrenochaeta inflorescentiae* culture-collection CBS:119222 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence [EU552153.1]	99%	99%	MA6024	KY454757
	NS5/NS8	*Pyrenochaeta sp.* GMG_PPb7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence [FJ439593.2]	99%	99%		KY454759
X1	ITS1/ITS4	*Purpureocillium lilacinum* [KJ862077.1]	99%	100%	MA6022	KY454763
G	ITS1/ITS4	*Penicillium canescens [AF033493.1]*	99%	99%	MA6023	KY454764

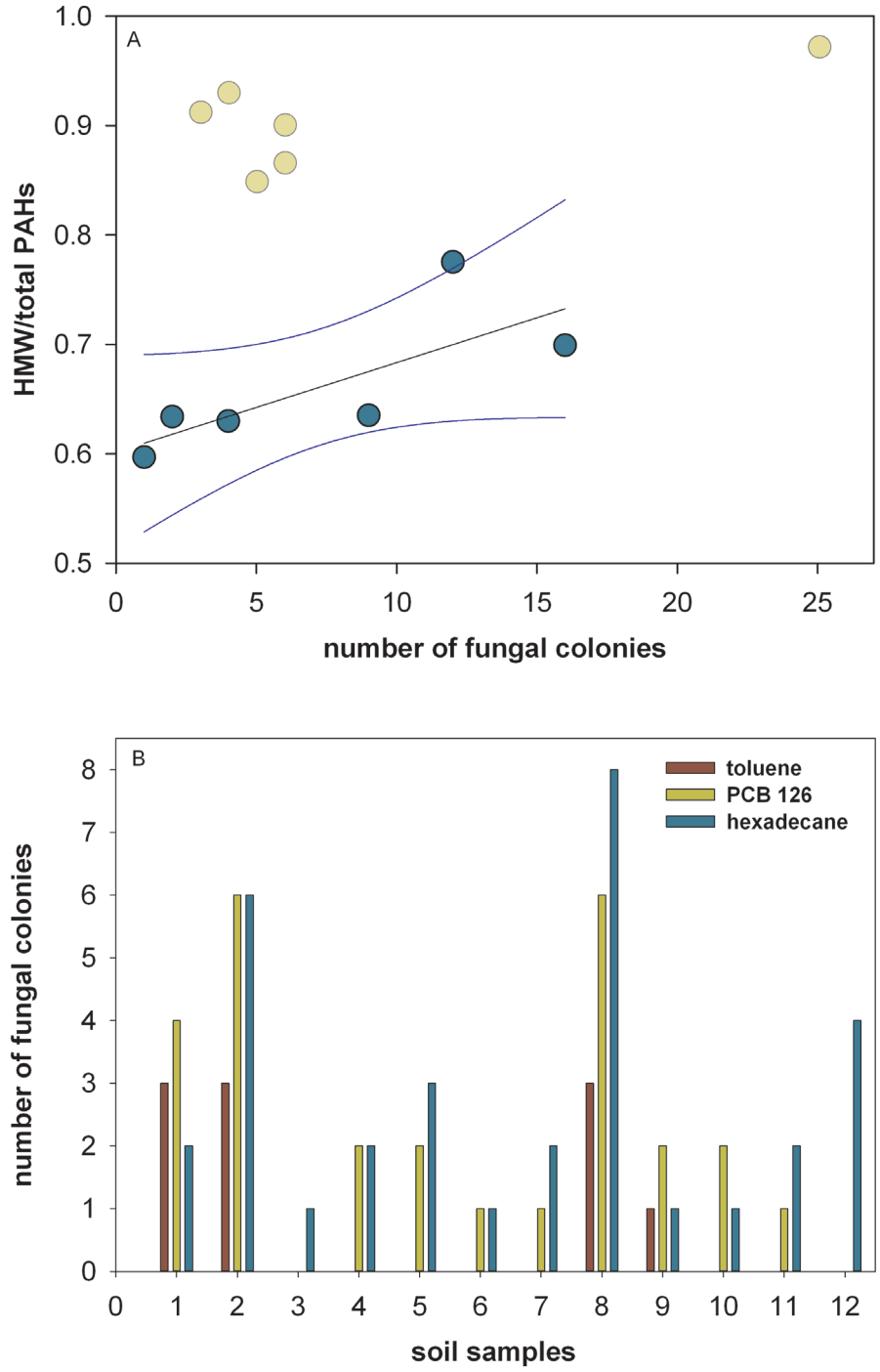

Figure 1. Regression model of PAH ratio against fungal colonies and fungal colonies isolated from each sample. **A:** Ratio of high molecular weight (HMW) PAHs to total PAHs on the *y*-axis against the total number of fungal colonies (x-axis) was plotted. A linear regression was done (black line) using the samples with low HMW/total PAH ratio (blue dots) with a confidence interval of 95% (blue line). The excluded samples with high HMW/total PAH ratio (indicating high age) was colored in green and excluded from the linear regression. **B:** Number of fungal colonies isolated from each sample grown on toluene (red), PCB 126 (green) and hexadecane (blue).
Abbildung 1. Regressionsmodell des PAK-Verhältnisses gegen die Anzahl der Pilzkolonien und der Gesamtanzahl der Pilzkolonien isoliert von den verschiedenen Proben. **A:** Verhältnis der hochmolekulargewichtigen (HMW) PAKs gegen die Gesamtanzahl der PAKs auf der y-Achse geplottet gegen die Gesamtanzahl der isolierten Pilzkolonien auf der x-Achse. Eine lineare Regressionsgerade wurde geplottet (schwarze Linie) mit Proben mit niedrigem HMW/total PAK Verhältnis (blau, runde Symbole) und ein Konfidenzintervall von 95 % berechnet (blaue Linie). Proben mit hohem HMW/total PAK-Verhältnisses wurden nicht in die lineare Regression genommen (grüne Punkte). **B:** Anzahl der Pilzkolonien isoliert von jeder Probe, welche in Anwesenheit von Toluol (rot), PCB 126 (grün) und Hexadekan (blau) gewachsen sind.

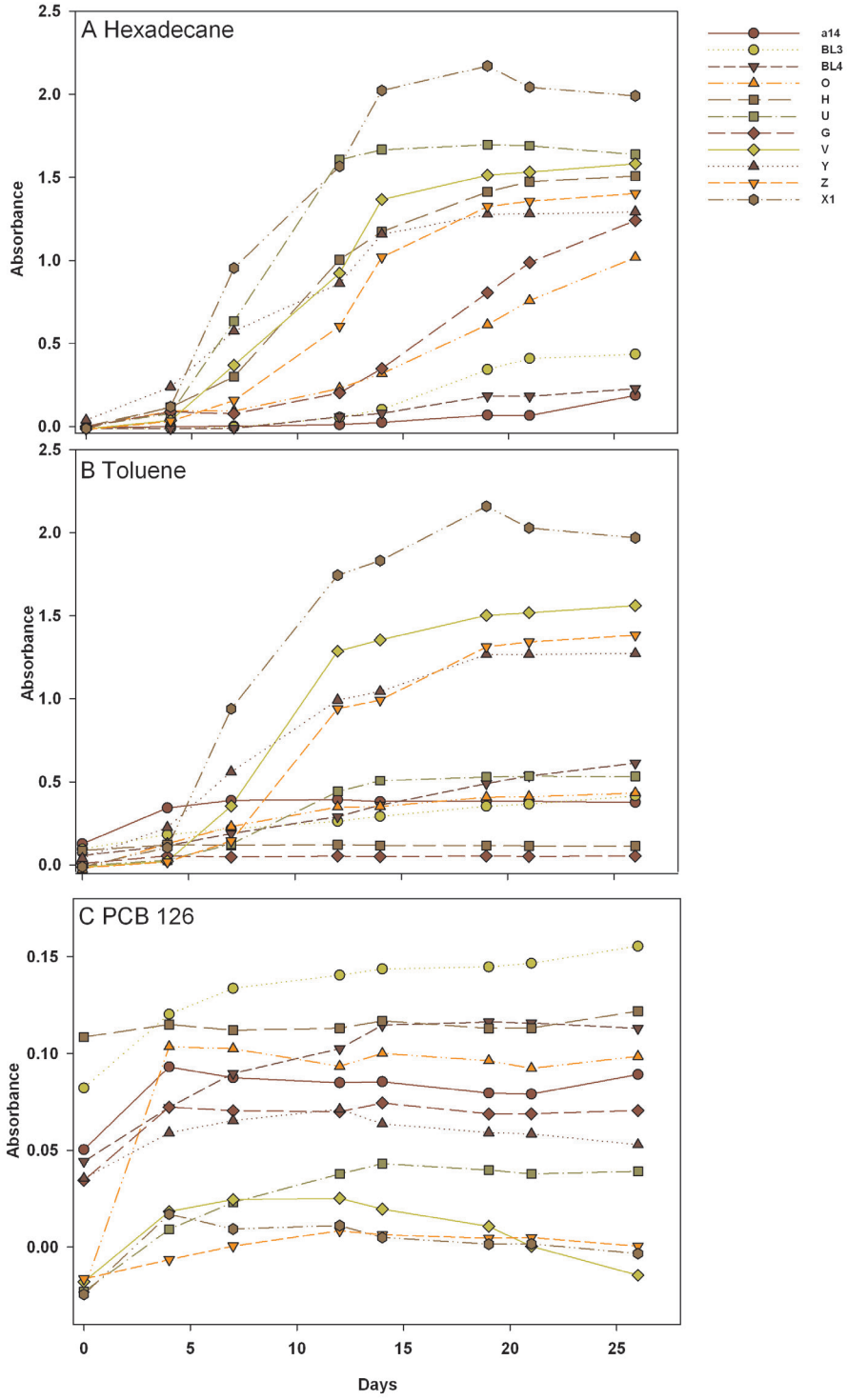

Figure 2. Fungal growth curves. Growth curves of the 11 selected fungal strains for identification grown in the presence of **A** hexadecane, **B** toluene and **C** PCB 126. Growth was measured through detected changes in the OD 700 using a Tecan reader.
Abbildung 2. Wachstumskurven der Pilzstämme. Wachstumskurven der 11 identifizierten Pilzstämme, die in Gegenwart von **A** Hexadekan, **B** Toluol und **C** PCB 126 gewachsen sind. Das Wachstum wurde durch Änderung der OD 700 mit Hilfe eines Tecan Readers detektiert.

Table 2. GC Results: Measured US EPA PAH concentrations and standard deviations of the 12 soil samples (S1-12). Values are presented as mg/kg dry weight and standard deviations were calculated (mg/kg dryweight±standard deviation)
Tabelle 2. GC-Ergebnisse: Gemessene US EPA PAK-Konzentrationen und Standardabweichungen der 12 Bodenproben (S1-12) sind in der Tabelle dargestellt. Die Werte sind also mg/kg Trockengeweicht dargestellt und die Standardabweichung wurde berechnet (mg/kg Trockengewicht±Standardabbweichung)

sample	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Naphtalene	5.8±1.2	4.8±1.0	12.8±2.6	9.4±1.9	6.8±1.4	31.5±6.3	0.1±0.02	<0.3(0.0)	<0.3 (0.0)	0.1±0.01	<0.3(0.0)	<0.3(0.0)
Acenaphthylene	5.8±1.2	1.8±0.4	<2.7(0.00)	0.9±0.2	0.9±0.2	5.2±1.0	0.1±0.02	<0.3(0.0)	0.4±0.1	0.2±0.03	0.7±0.1	0.3±0.1
Acenatphene	6.9±1.4	4.3±0.9	11.2±2.2	8.5±1.7	6.8±1.4	24.9±5.0	0.6±0.1	0.4±0.1	0.4±0.1	0.1±0.02	<0.3 (0.0)	0.3±0.1
Flurene	10.1±2.0	6.3±1.3	14.8±3.0	10.3±2.1	8.3±1.7	34.3±6.9	0.5±0.1	0.4±0.1	0.4±0.1	0.1±0.02	0.5±0.1	0.6±0.1
Phenanthrene	55.20±11.0	28.3±5.7	70.2±14.0	48.3±9.7	36.5±7.3	154.0±31.0	5.9±1.2	4.4±0.9	4.1±0.8	1.0±0.2	6.4±1.3	5.7±1.2
Anthracene	22.5±4.5	11.4±2.3	22.2±4.4	18.1±3.6	14.4±2.9	67.7±13.5	1.4±0.3	1.2±0.2	1.6±20.3	0.4±0.01	2.1±0.4	1.4±0.3
Fluoranthene	78.0±15.6	29.1±5.8	48.2±9.6	39.0±7.8	30.8±6.2	141.0±28.0	7.5±1.5	12.6±2.5	10.0±2.0	2.9±0.6	17.5±3.5	9.8±2.0
Pyrene	77.8±15.6	30.4±6.1	47.9±9.6	41.6±8.3	32.2±6.4	108.0±22.0	7.5±1.5	24.4±4.9	10.0±2.0	2.3±0.5	14.5±2.9	7.7±2.0
Benz[a]anthracene	36.5±7.3	13.5±2.7	19.9±4.0	16.6±3.3	13.0±2.6	64.3±12.9	4.7±1.0	23.5±4.7	9.8±2.0	1.9±0.4	9.8±2.0	5.9±1.2
Chrysene	37.4±7.5	13.3±2.7	20.7±4.1	15.8±3.2	12.5±2.5	68.6±13.7	5.9±1.2	27.3±5.5	11.0±2.2	2.2±0.4	10.2±2.0	7.1±1.4
Benzo(b) fluoranthene	33.0±6.6	10.4±2.1	14.4±2.9	10.9±2.2	9.0±1.8	36.1±7.2	6.5±1.3	28.1±5.6	11.9±2.4	1.9±0.4	9.1±1.8	7.1±1.4
Benzo(k) fluoranthene	26.6±5.3	9.5±1.9	12.7±2.5	10.6±2.1	8.5±1.7	45.3±9.1	4.2±0.8	33.0±6.6	10.4±2.1	1.7±0.3	8.0±1.6	5.0±1.0
Benzo(a)pyrene	30.6±6.1	10.9±2.2	14.3±2.9	11.9±2.4	9.76±2.0	44.7±8.9	5.0±1.0	27.1±5.4	9.8±2.0	1.8±0.4	8.5±1.7	5.1±1.0
Indeno(1,2,3-cd)pyrene	21.3±4.3	7.0±1.4	8.5±1.7	8.3±1.7	6.0±1.2	20.1±4.0	3.1±0.6	19.6±3.9	8.2±1.6	1.7±0.3	5.2±1.0	2.8±0.6
Dibenzo(a,h) anthracene	5.9±1.2	1.7±0.4	<2.7(0.0)	1.6±0.3	1.3±0.3	4.8±1.0	1.1±0.2	5.5±1.1	2.0±0.4	0.5±0.1	1.2±0.2	0.7±0.1
Benzo(g,hi,i) perylene	19.6±3.9	6.5±1.3	7.8±1.6	6.4±1.3	5.2±1.1	17.2±3.4	3.4±0.7	22.7±4.5	7.2±1.5	1.8±0.4	4.2±0.8	2.7±0.5
sum	472.9±94.6	189.4±38.0	325.6±65.1	258.1±51.7	202.0±40.4	867.8±173.9	57.4±11.5	230.2±46.0	97.2±39.5	20.6±4.0	97.6±19.4	62.2±12.9

Ochroconis longiphorum (CBS 435.75) was isolated from the Canadian soil underneath the asphalt of a car park (Samerpitak et al., 2014), but also from the soil contaminated by a leaking diesel tank (Andreolli et al., 2016). *Ochroconis longiphorum* was formerly also described as *Scolecobasidium longiphorum*. In the genus *Ochroconis*, opportunistic infections of cold blooded vertebrates are reported but also for some thermophilic species warm-blood vertebrates might be a susceptible host (Samerpitak et al., 2014).

Pyrenochaeta inflorescentiae showed good growth in the presence of hexadecane and toluene. The reference listed in the Blast result (GenBank: EU552153.1) is deposited at the strain collection of the CBS-KNAW fungal biodiversity Center (CBS 119222), originating from Cape Town, from a senescent flowerhead. No degradation of contaminants of *P. inflorescentiae* was reported yet, but *Pyrenochaeta* sp. DS3sAY3a, was isolated from passive coal mine drainage treatment systems in central Pennsylvania, where toxic metals are removed from the drainage water (Santelli et al., 2010). Zeiner et al. (2016) concluded that *Pyrenochaeta sp.* could contribute to lignocellulose degradation by wood-rot Basidiomycete fungi using cellulases and hemicellulases together with redox-active accessory enzymes in their cellulose-degrading capacity. This degrading capacity may be based on the direct enzymatic carbohydrate breakdown as well as indirect carbon oxidation via Fenton-based hydroxyl radical formation (Zeiner et al., 2016). Results from our study show that *P. inflorescentiae* can live in the presence of contaminants and might even be able to degrade them not only through co-metabolism with wood-rot fungi.

Table 3. Results of the second microtiter plate screening: Number of colonies of having the same growth pattern of all 93 fungal isolates on toluene, hexadecane and PCB 126. + = good growth, ~ = very slow growth and - = no growth. Hex = hexadecane, Tol = toluene, PCB = PCB 126
Tabelle 3. Ergebnisse des zweiten Mikrotiterplatten-Screenings: Anzahl der Kolonien mit demselben Wachstumsmuster innerhalb der 93 isolierten Kolonien in Gegenwart von Toluol, Hexadekan und PCB 126. + = gutes Wachstum, ~ = sehr langsames Wachstum und - = kein Wachstum. Hex = Hexadekan Tol = Toluol, PCB = PCB 126

colony numbers	Hex	Tol	PCB
21	+	+	-
14	+	+	~
9	+	+	+
8	+	-	-
6	+	~	~
5	+	-	~
4	-	-	-
3	~	+	-
3	+	~	-
2	-	+	-
2	~	-	-
2	~	-	~
2	~	~	~
2	~	+	~
2	+	~	+
2	~	~	+
1	-	~	-
1	-	-	~
1	-	~	~
1	~	~	-
1	-	~	+
1	~	~	~

Table 4. Growth pattern of the chosen fungal isolates for sequencing: + = good growth, ~ = very slow growth and - = no growth. Hex = hexadecane, Tol = toluene, PCB = PCB 126
Tabelle 4. Wachstumsmuster der zum Sequenzieren ausgewählten Pilzisolate: + = gutes Wachstum, ~ = sehr langsames Wachstum und - = kein Wachstum. Hex = Hexadekan Tol = Toluol, PCB = PCB 126

No	Hex	Tol	PCB
α 14	+	+	~
O	+	+	-
U	+	+	+
V	+	+	-
X 1	+	+	-
Y	+	+	-
Z	+	+	~
BL 3	+	+	+
BL 4	~	+	~
G	+	-	~
H	+	-	~

The isolation of *Roussoella intermedia* in this study pointed out as there is no report on the degradation of contaminants, but still the strain grew in the presence of all three contaminants. *Roussoella intermedia* was isolated from bamboo tree trunks in Papua New Guinea (CBS 170.96) and Hong Kong (ZHOU et al.) and it was isolated from north temperate lakes (Fallah-Moghaddam, 1999). Ligninolytic fungi as *Roussoella* are mostly wood degraders and they can be enriched through lignin-rich additions such as tree branches to soil (Bamforth and Singleton, 2005). Lignocellulosic material, which was colonized by the fungal strain originally, may have been added to the soil and the ligninolytic enzymes for wood degradation may have helped to degrade PAHs and the other contaminants tested.

Penicillium janthinellum shows significant growth in the presence of hexadecane, toluene and PCB 126, although the latter at a lower rate (Figure 2c). *Penicillium janthinellum*, renamed *Penicillium simplicissimum* was isolated from S 1 with the highest concentration of Pyrene, Chrysene and Benzo[a]pyrene. This is in line with literature where degradation of Pyrene, Chrysene, Benzo[a]pyrene (Cerniglia, 1997) by this strain is reported. *Penicillium janthinellum* has the ability to immobilize aluminum (Leitão, 2009) and was isolated from extremely acidic soil (Hujslová et al., 2017). Boonchan et al. (2000) suggest that *P. janthinellum* needs a bacterial co-culture to mineralize benzo[*a*]pyrene as a sole carbon and energy source. It is reported to degrade the aliphatic and aromatic fractions of crude oil (Oudot et al., 1987), oxidize chrysene (Kiehlmann et al., 1996) and April et al. (1999) report that 31% of the aliphatic fraction of Norman Well crude oil was degraded. Compared to other isolated fungal strains, it is not reported to be pathogenic for human and therefore, is a good candidate for bioremediation purposes.

Another good degrader for hexadecane might be *Penicillium canescens*. *Penicillium canescens* showed increased OD values in the presence of hexadecane and slightly with PCB. It was described by Say et al. (2003) as biosorbent for heavy metals from aqueous solutions and was therefore

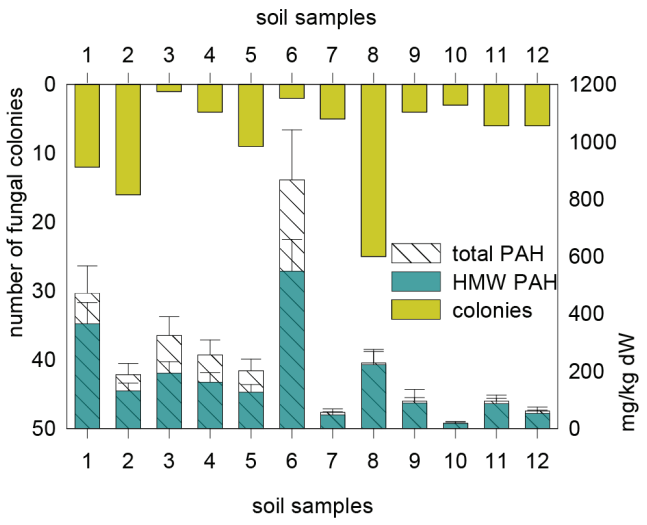

Figure 3. PAH concentrations and number of fungal colonies isolated from each sample. Top, left axis (green): Total sum of colonies isolated in the presence of toluene, PCB 126 or hexadecane, and wells containing glucose. Bottom, right axis: total concentration (pattern) and proportion of HMW PAHs (blue). X-axis presents the different samples.
Abbildung 3. PAK-Konzentrationen und die Anzahl der von jeder Probe isolierten Pilzkolonien. Obere, linke Achse: grün: Gesamtanzahl der Kolonien isoliert in Anwesenheit von Toluol, PCB 126 oder Hexadecan als einzige Kohlenstoffquelle und Näpfchen mit Glukose. Untere, rechte Achse: Gesamtkonzentration (Muster) und Proportion an hochmolekulargewichtigen PAKs (blau). Die x-Achse zeigt die verschiedenen Proben.

suggested as a strain for the cleanup of the environment, especially the aqueous systems. Further, it was shown to degrade dibenzofuran (Hammer et al., 2001). Garon et al. (2004) reported the isolation of the strain from a heavily PAH contaminated soil. Interestingly, it was isolated from S6, the sample with the highest amount of PAHs.

Doratomyces purpureofuscus was described by Bello Mouhamadou et al. (2013) as being isolated from the PCB contaminated soil and was shown to be able to degrade a mixture of 7 PCBs. It shows very slow growth with PCB 126 in this screening but increased growth in the presence of toluene. This non-pathogenic strain is successfully growing also in presence of hexadecane. Interestingly, 7 out of the 11 strains are opportunistic pathogens or have a close pathogenic strain within the genus. In the genus *Roussoella*, there is one reported opportunistic pathogen, *Roussoella percutanea* (Ahmed et al., 2014). In the genus *Pyrenochaeta*, the strain *Pyrenochaeta romeroi* (Yadav et al., 2015; Kulkarni et al., 2016) is reported to cause subcutaneous phaeohyphomycosis in transplant or diabetes patients (Yadav et al., 2015; Kulkarni et al., 2016). *Purpureocillium (Paecilomyces) lilacinum* is also reported as an opportunistic pathogen (Luangsa-Ard et al., 2011). This is in line with the hypothesis of *Prenafeta* et al. (2006), which suggests a linkage of microbial degradation potential and pathogenicity that might be due to the similarity of chemical structures of several brain components resembling alkylbenzene and lignin biodegradation intermediates.

4. Conclusion

In our study, we present the successful usage of a microtiter plate screening method for isolating the fungal strains from PAH contaminated soil. Microbiota originating from the contaminated soil is reported by Garon et al. (2004) to degrade contaminants better than the collection of originating reference strains. Our study combines the advantage of the quick, high throughput screening method and the selective pressure of contaminated soil. The method was proven successful not only of being able to isolate 93 cultivable colonies but also 9 strains could be identified that are connected to the degradation of organic contaminants. The 11 identified strains could potentially be applied to bioremediation or biofilter systems. The strains are isolated from specific sites in Austria and should be well adapted for similar contaminated sites.

In 6 out of 12 samples, a link between the proportion of HMW to total PAH concentration and the total number of isolated fungal communities could be observed. Additionally, 20% of the strains were not able to grow the presence of the contaminants as a pure culture, which might be explained by mutualism with other fungal strains, other members of the soil microbiota, lack of substrate from the soil inoculant or difference in toxic pressure from the contaminant substrate.

The identified 11 strains can be used as a starting point for detailed research on carbon utilization and degradation performance. Especially, the use of the contaminants as sole carbon source without producing toxic cometabolites or additional carbon sources such as dibutyl phthalate from the microtiter plate material needs to be confirmed. Future work will have to address the toxicity of intermediate products, fate, degradation pathways, enzymes used and up and downregulated genes. While for ligninolytic fungi, a lot of studies concerning the enzymes involved in PAH degradation are available; for non-ligninolytic fungi, this information is lacking (Marco-Urrea et al., 2015). The correlation

of 7 strains with the suggestion of Prenafeta et al. (2006), proposing a link between the capabilities of fungal strains to degrade contaminants and being neurotropic agents for warm-blooded vertebrates, has to be further investigated and might also help to understand fungal pathogenicity. Therefore it is of utmost importance to understand fungal degradation mechanisms and strains contributing to bioaugmentation to reduce the risk of spreading pathogenic microbes. More studies are needed to enable an effective usage of non-ligninolytic fungi, understand their degradation mechanisms, interaction with other microorganisms in degradation application and anticipate new effective technologies.

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