Potential uptake and disintegration of PFAS 22 by the fungi *Pleurotus ostreatus* and *Trametes versicolor*

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Titel	Potential uptake and disintegration of PFAS 22 by the fungi *Pleurotus ostreatus* and *Trametes versicolor*
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Introduction

As a society, we have made it a crucial task to recycle our wastewater. Urban drains, constructed freshwater basins, and even the water flushed down the toilet, are all directed to water purification plants. Here the water undergoes a meticulous cleaning process before being released into the ocean. Sewage sludge is a material produced as a by-product during the treatment of industrial and urban wastewater. It is a semi-solid residue composed of organic waste, such as fecal matter, algae and dead and living microorganisms. This composition makes sewage sludge rich in essential organic nutrients like nitrogen (N), potassium (K), and phosphorus (P), making it a valuable resource for sustainable soil fertilizer. However, the use of sewage sludge as fertilizer is subject to environmental restrictions, due to the presence of harmful contaminants like heavy metals and per- and polyfluoroalkyl substances (PFAS). Although these substances have been largely banned in about a decade, they still impact the chemical composition of sewage sludge. PFAS's have been nicknamed "forever chemicals" because of their incredibly persistent structure. PFAS are characterized by their very strong carbon-fluoride bonds, which nearly render them undegradable. The bioaccumulation of PFAS has among other things been linked to carcinogenicity and endocrine disruption[1]. Therefore, the Danish Environmental Protection Agency has set certain criteria for PFAS levels in sewage sludge used as fertilizer. For instance, the limit for PFAS-22 is 50 µg/kg (in dry matter)[2]. Sewage sludge that exceeds the maximum limit is disposed of by unsustainable methods like incarceration. Thus, it is crucial to search for solutions that optimize the sustainable use of sewage waste.

[1] Sludge and the circular economy- the impact of PFAS. (s.d.). *Eurau*, s. 1-3.
[2] Jensen, J. (2023, March). *Derivation of cut-off values for PFAS in sewage sludge* (2232) [Report]. The Danish Environmental Protection Agency

Purpose

This synopsis explores the potential for the uptake and degradation of per- and polyfluoroalkyl substances (PFAS), particularly PFAS-22, in sewage sludge. Utilizing ligninolytic enzymes produced by the white-rot fungi *Trametes versicolor* and *Pleurotus ostreatus*. Enzymatic mechanisms are the hypothesis this synopsis relies on to lay the grounds for utilization of white-rot fungi to degrade xenobiotics like PFAS. Thus, reducing contamination levels to meet environmental standards. The synopsis provides an experimental design that proposes the use of sewage sludge and sawdust as substrate for the two fungi. In the design the PFAS levels of both substrate and fungi will be quantified at set periods in time, to determine if the fungi affect PFAS-levels. The findings of said design could provide insights into the general role of fungal species' enzymatic activity in mitigating environmental pollution. The hypothesis has been applied in some completed studies, in one of which an isolated laccase strain from *P. ostreatus* was shown to degrade PFOA by 157 days in the presence of 1-hydroxybenzotriazole as a redox mediator. Moreover, they obtained 40% degradation after 140 days in the presence of soybean meal and laccase in a soil slurry.[3] My experimental design differs from this, the enzymes aren't isolated, and the degradation of substrate depends on the variety of enzymes secreted by two fungi, potentially obtaining a higher degradation

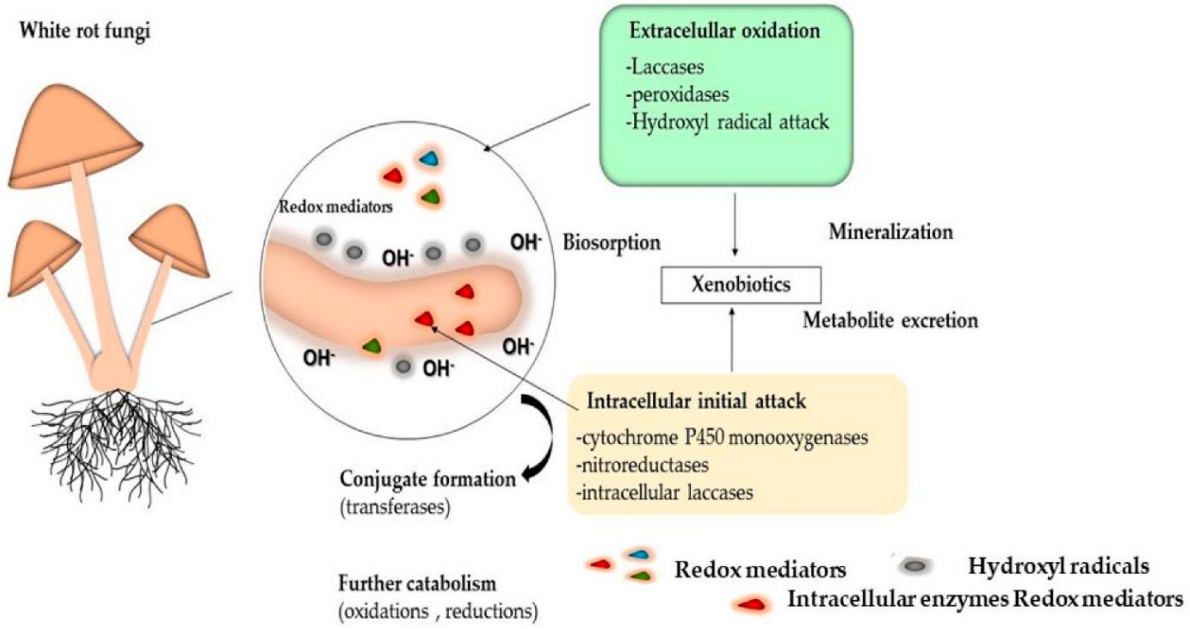

Figure 1 - White rot fungi mechanisms and enzymes associated with the initial intracellular attack and extracellular oxidation [4]

[3] Luo, Q. (2015). Laccase catalyzed degradation of perfluorooctanoic acid

[4] Torres-Farradá, G. (2024). White Rot Fungi as Tools for the Bioremediation of Xenobiotics: A Review. *Journal of Fungi*, (1), s. 13-16, s. 24-30.

Fungi first derive their nutrients by extracellular digestion, they secrete enzymes trough the hyphae[5] cell wall to oxidate substrate. The oxidation breaks down the substrate into smaller components that can be absorbed by the fungi.[6] When it is absorbed, the substrate is faced with a range of intercellular enzymes, seen as the red triangles inside the hyphae. The fungi *Trametes versicolor* and *Pleurotus ostreatus* are both saprotrophic white-rot fungi, characterized by their ability to secrete lignin modifying enzymes. They grow on decaying wood, thus their natural growth substrate consists of the lignocellulosic compounds, lignin, cellulose and hemicellulose, which are all complex organic polymers. White-rot fungi are unique in their ability to degrade lignin, because of their secretion of a variety of ligninolytic enzymes, such as laccase, lignin peroxidase, versatile peroxidase and manganese peroxidase[7]. Ligninolytic enzymes are extracellular, and operate through free radical mechanisms[8], enabling them to degrade a wide variety of structurally diverse xenobiotic compounds.[9] The peroxidases facilitate both direct and indirect oxidation outside the cell, showcased by the green box. The indirect reactions are mediated by redox agents like veratryl alcohol and Mn^{2+} [10], while the direct oxidation is mediated by hydroxyl radicals, seen as grey dots on figure 1.

Trametes versicolor and *Pleurotus ostreatus*

The mushrooms *P.ostreatus* and *T.versicolor* are chosen specifically for the enzymes that they secrete through the mycelia and the fruit body, but also because of the similarity of their optimal environment.

Phase	Temperature	Duration	Humidity	Light	CO2	Fresh air
Incubation	24°C	14 - 21 days	-	/	>5,000 ppm	1 time per hour
Initiation of primordia	10 - 24°C	1 to 3 weeks	95 - 100%	500 - 2,000 lux	400-800 ppm	5 - 7 times per hour
Fructification	18 - 24°C	45 - 70 days	85 - 90 (95)%	500 - 2,000 lux	500 - 1,000 ppm	5 - 7 times per hour

Figure 2 -Trametes versicolor culture settings[11]

[5] Hyphae: the root-like branches that constitute the mycelia
[6] Moore, D. (s.d.). *Ecology of fungi*. Britannica.
[7] Nagai, M. (2007). Isolation and characterization of the gene encoding a manganese peroxidase from Lentinula edodes: Mycoscience. *ScienceDirect*, s. 125-130]
[8] Free radical: an atom with an unpaired electron
[9] Torres-Farradá, G. (2024)
[10] Shrivastava, R. (2005 , April). Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: enzymology and mechanisms involved. *Pubmed*, (4)
[11] Stamets, P. (1993). *Growing Gourmet and Medicinal Mushrooms*

Phase	Temperature	Duration	Humidity	Light	CO2	Fresh air
Incubation	20-24°C	2-3 weeks	-	/	/	/
Initiation of primordia	10-15°C	4-7 days	95-100%	600-1000 lux	500-800 ppm	4 to 8 volume/h
Fructification	10-20°C, ideal 15°C	5-10 days	85-95%	600-1000 lux	500-800 ppm	4 to 8 volume/h

Figure 3 - Pleurotus ostreatus culture settings[12]

As seen on figure 2 and 3 showcasing the optimal environment of the fungi, the estimate for the optimal humidity is the same for both mushrooms, and the range of the other conditions such as light, CO_2 and airing is largely overlapped. This ensures that the two fungi can grow optimally under the same conditions. The main difference between *T.versicolor* and *P.ostreatus* lies in the length of their lifespan. The technicalities of the phases in the lifecycle of the two fungi is important when choosing the time to collect data in an experiment showcasing the potential uptake of PFAS. The incubation phase is where the spores develop into mycelium. As shown in figure 1 it is through the hyphae of the mycelium that enzymes are secreted onto substrate. Then comes the initiation of primordia[13], which is the first development of fruit bodies. It is in this stage that it would be sensible to conduct chemical analysis of the substrate and fungi to quantify PFAS. The development of primordia in *T.versicolor* is according to the figure significantly slower, but since the incubation length of the fungi is the same, it should suffice to collect the first samples on day 28. The last phase, the fructification, is where the two species overlap the least in their lifecycles. The fructification of *P. ostreatus* and *T. versicolor*, are as seen on the figures, 5 - 10 days and 45 -70 days respectively. This does fortunately not pose a problem for the experiment if the samples that are to be collected from *P. ostreatus* are collected before the fungi withers. For *T. versicolor* the samples will be collected on day 60, where the fungi according to figure 2 should have reached its final form. The fact that the lifecycle of *Trametes versicolor* is significantly shifted from the one of *P. ostreatus* creates an interesting component in the experimental design. There are multiple possible outcomes if the two fungi are planted in one space. They could either compete with one another or develop a symbiotic relationship, either of these scenarios could lead to a greater disintegration of PFAS than in the spaces where only one type of fungi is placed.

[12] Ibid.

[13] Primordia: Little knobs that are the first indication of a fruit body

Methods

Liquid Chromatography Tandem Mass Spectrometry is the ideal method for detecting and quantifying per- and polyfluoroalkyl substances (PFAS) due to its high sensitivity and ability to handle complex mixtures. This is essential for this synopsis because the experiment involves quantification of PFAS 22 in the multi-contaminated sewage sludge and fruit body of the fungi.

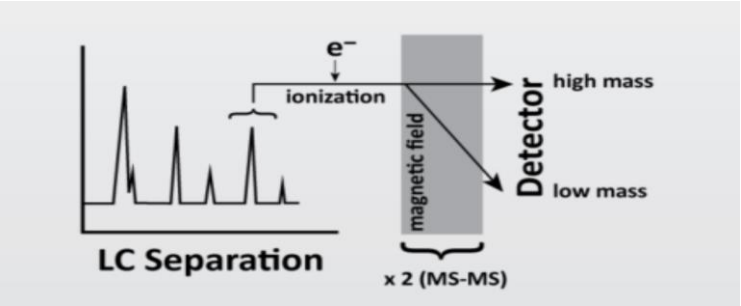

Figure 4 - liquid chromatography tandem mass spectrometry[14]

The first part of the process is shown on figure 4 with the LC separation chromatogram. The peaks in the chromatogram represent different compounds being separated by a reverse phase LC column[15]. Each peak corresponds to a different compound. The program making the diagram will be calibrated with the known value of the analyte, in this case the individual PFAS found in the group of PFAS 22. These separated compounds are moved via a solvent consisting of water and acetonitrile, mixed with ion pairing agents such as trifluoroacetic acid, formic acid or heptafluorobuteric acid[16]. After leaving the LC, the compounds are subjected to electrospray ionization (ESI), this is essential because the mass-spectrometer doesn't detect neutral particles. In the diagram, the "e^-" represents the addition or removal of electrons, creating charged ions. Then a mass-spectrometer applies a magnetic field to separate the ions based on their mass-to-charge ratio *(m/z)*. The diagram illustrates how ions of different masses are deflected by the magnetic field at different angles. Ions with higher mass-to-charge ratios are deflected less, while ions with lower mass-to-charge ratios are deflected more. The term MS-MS refers to tandem mass-spectrometry, where there are two stages of mass analysis. The first stage (Q1) selects the parent ion[17] (e.g., PFAS 22), while the second stage (Q3) analyzes the fragment ions produced after the parent ion is fragmented in the collision cell. Finally, in the Detection stage, the separated ions hit the detector, which measures their intensity and abundance. This is translated into a mass spectrum that allows the detector to differentiate between ions of varying masses, thereby quantifying the concentration of PFAS 22 by comparing the detected signals to calibration standards. In this experiment the LC/MS/MS process will be conducted by a second party like Eurofins, they charge 2.000DKK pr. sample run.

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[14] Anonym. (s.d.). *Basics of LC/MS*
[15] Reverse LC column: The most common separation method in liquid chromatography
[16] Solvents for LC-MS. (s.d.). *Thermo Fischer Scientific*
[17] Parent ion: Ion that dissociates to a smaller fragment ion

Pot- experiment

The simplest way to evaluate the potential uptake and disintegration of perfluoroalkyl substances (PFASs), by the mushrooms *Pleurotus ostreatus* and *Trametes versicolor* in practice, is to conduct a pot experiment. This minimalistic approach is chosen to ensure the best control over variables and natural variation in responses. Both mushroom cultures in the experiment are bought as sawdust-spawn, which is the mycelia-colonized base that will be added to the "bulk substrate". In this experiment, the mushrooms use sewage sludge for its nutritional properties, and thus they will potentially degrade PFAS as nutritional matter. But to ensure that the mushrooms grow optimally, the bulk substrate will consist of 2/3 sterilized sawdust. The sawdust provides the mushroom with lignocellulosic compounds. These materials mimic the natural habitat of the two fungi and create a moist but solid substrate optimal for the mycelia's expansion. The lab environment will be installed to match the overlapping optimal settings of the fungi, shown in *figure-2* and *figure-3*.

In total the experiment has 20 pot-containers consisting of 5 variations each with 4 replicas, each of the pots with a volume of *1 L*. The experiment requires *16* L of PFAS polluted substrate with known concentration[18], and *4 L* of non-polluted substrate. To speed up the LC/MS/MS process of the samples later in the experiment, the two substrate variations are crushed into separate homogenous masses. The polluted substrate is distributed between *4 x 4* containers. Four of the containers with polluted substrate will be a positive control. They will not be colonized by fungi and serve to show whether and to what extend PFAS degrades independently throughout the experiment. Another 8 pots of polluted substrate will be inoculated with sawdust spawn of *P. ostreatus* and *T. versicolor* separately, while the remaining 4 pots are colonized by both fungi simultaneously. The ratio of sawdust spawn to substrate is 10% in the containers with one mushroom culture, whereas it is *5%* of each spawn in the containers with both cultures. The remaining unpolluted substrate serves as a negative control. Samples will be collected from the 20 containers three times over the course of two months, on day 0, 28, and 60.

LC/MS/MS determines the mass of PFAS22 in fungi and substrate. The samples for the LC/MS/MS will be gathered through pooling, a process where samples from each replica of the five variants are unified into one sample. The unified sample provides a median of the four replicas, which reduces the final cost of the project. Furthermore, the budget only allows chemical analysis of substrate and fungi in the beginning and end of the experiment *(day 0 and 60)*. The pooled samples that are to undergo LC/MS/MS will be conducted as shown on the first of the following figures.

[18] The concentration will be known because the contaminated sewage sludge exceeds the environmental criteria, it is tested on the water treatment plant.

LC/MS/MS			
Data collection	**Treatment Group**	**PFAS 22 Analysis**	**Poled Samples**
Day 0	*Negative Control (substrate without PFAS)*	*1 Substrate*	*1*
	Positive Control Substrate	*1 Substrate*	*1*
Day 60	*Negative Control (substrate without PFAS)*	*1 Substrate*	*1*
	Positive Control Substrate	*1 Substrate*	*1*
	Substrate + Fungi 1	*1 Substrate, 1 Fungi*	*2*
	Substrate + Fungi 2	*1 Substrate, 1 Fungi*	*2*
	Substrate + Fungi 1 + Fungi 2	*1 Substrate, 2 Fungi*	*3*
Total			**11**

** The Substrate is a mixture of 66% sawdust and 33% sewage sludge contaminated with PFAS.*

BUDGET			
Item	**Quantity (Batch)**	**Cost per Batch (DKK)**	**Total Cost (DKK)**
PFAS 22 Analysis (Substrate)	*7 samples*	*2000*	*14000*
PFAS 22 Analysis (Fungi)	*4 samples*	*2000*	*6000*
P. ostreatus Spawn	*1 batch*	*108*	*108*
T. versicolor Spawn	*1 batch*	*108*	*108*
Sawdust	*1 batch*	*50*	*50*
Sewage Sludge (Waste Material)	*20 L*	*0*	*0*
Total			**22266**

Figure 5 - LC/MS/MS overview and project costs

As seen in the top figure, 11 samples are to be analyzed when collecting data purely from the beginning and the end of the experiment. The data of the poled samples collected in between day 0 and 60 will be frozen down. If other funding, can be found then the frozen material from the third time period can be analyzed. The given project budget covers 20.000 DKK, thus the exceeding 2.266 DKK, which is the cost of one LC/MSMS, sawdust batches and fungi spawn, will be provided by personal funds. The result of the experiment is obtained by setting the data of day 0 and 60 into the following formula

$$D_{PFAS22} = C_{PFAS22\,(day\,60)} - C_{PFAS22\,(day\,0)}$$

Equation 1- D:degredation, C: concentration

It should be made clear that the substrate is analyzed to find out whether and how much PFAS22 is degraded during a period of 60 days, while the fungi are analyzed to ensure that the toxins have been degraded and not simply transferred to the fruit body. Fungi samples will only be analyzed on day 60 because due to the fungi being spawn on day 0. This leaves the experiment without a baseline for PFAS22 in fungi. To have had a baseline, there would be established a 6'th variation of the experiment with the two fungi inoculated separately and together in unpolluted substrate, but that would be a cost addition of 6000 DKK. Therefore, this design assumes the baseline concentration of PFAS in unpolluted substrate is zero.

Conclusion

The main concern for this design is the possible negative impact of the bacteria present in sewage sludge. The bacterial flora in sewage sludge is unpredictable, as it is a waste material, and could possibly stunt the growth of fungi in the early stages. Another concern is the budget's impact on the data collection, the samples are only collected and pooled three times, while only the start and finish concentrations are analyzed. It would be optimal if the budget allowed more data-collection. The pooling of the data also masks the variability between the replicates, meaning the data obtained will be less detailed. The currents setup concludes whether the fungi, separately or together degrade PFAS22. If the experiment can conclude that the PFAS22 has been degraded, and not simply transferred to the fruit-bodies, the whole experimental setup would be eligible for use as fertilizer. Unless the degradation does not lower the PFAS concentrations to meet the environmental criteria. An eventual upscaling of this design would include several more data collections and LC/MS/MS analysis of all datapoints to provide a detailed graph featuring the degradation.

Acknowledgements

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