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Quantifying the degradative capabilities of the oyster mushroom, *Pleurotus ostreatus,* on the common herbicides Metolachlor, Acetochlor, and Cyanazine with LC-MS analysis

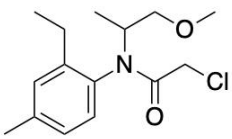
Abstract:

The focus of this study will be to analyze and compare how *Pleurotus ostreatus*, commonly known as the oyster mushroom, degrades long persistent pesticides. The study will be used to better understand how *Pleurotus ostreatus*, a culinary mushroom reacts and degrades long persistent herbicides, acetochlor, cyanazine, and metolachlor. This mushroom is a white-rot fungi and has previously been used to successfully degrade polyaromatic hydrocarbons. When pesticides are used, the remaining chemicals can be embedded in the food, soil, and water and be consumed by humans and animals, which will cause both detrimental effects on their cellular structure and bodily functions. The pesticides flowing into the groundwater allows for the water to be contaminated from the chemicals. Groundwater contamination can result in poor water quality, which can allow bacteria to grow in water storages and can result in various illnesses to those intaking the water, such as animals, plants, and humans, thus this scientific problem aligns itself well with the One Health initiative. Poor groundwater quality can lead to poor crop yield and can further contaminate food. We propose a solution to degrading the pesticides using *Pleurotus ostreatus*. To evaluate the efficacy of *Pleurotus ostreatus* on degrading the herbicides acetochlor, cyanazine, and metolachlor, samples of *Pleurotus ostreatus* will be spiked with each herbicide at three concentrations mirroring those found in polluted water. LC-MS analysis will be used to track the concentrations of each pesticide over a 5 week period as well as the identity of the degradates.

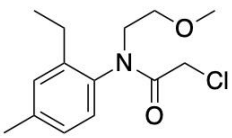
Section I

Agricultural practices can involve large quantities of pesticides, which may then remain on the surface of crops, be taken up into the crop through the crop's roots, or leech into the soil below the crops (Pesticides in Our Food System, 2021). **This study aims to better understand how culinary mushrooms react with agricultural pesticides.** According to a study titled "Pesticides in drinking water" (Syafrudin et al., 2021), an average of 2 million tons of pesticides were used each year, globally, to reduce weed, insect, and pest presence in crops. Even if these pesticides are never taken up by crops, they may still be consumed by people after being consumed by soil dwelling organisms, and from there, working their way up the food chain. Additionally, pesticides that leak into the soil of agricultural land act as a non-point pollution source, and have the potential to contaminate groundwater and deteriorate soil health by creating nutrient imbalances. When agricultural runoff is contaminated with chemicals commonly found in these pesticides aquatic life can become threatened, and organisms that do not die due to chemical exposure can still ingest the harmful toxins. Pesticide exposure has been linked to non-Hodgkin lymphoma in humans, as well as a contributing factor to birth defects in unborn babies (Beach, 2017). Furthermore, specific subclasses of pesticides, such as herbicides, can increase resistance of the targeted crops/weeds. This requires farmers to use greater quantities in the future to compete with the species' ability to grow.

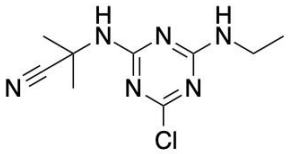
This study will focus on *Pleurotus ostreatus*' (Oyster mushroom) relationship to acetochlor, cyanazine, and metolachlor pesticides. Oyster mushrooms are a type of white-rot fungi and have been successfully used to degrade polyaromatic hydrocarbons. These pesticides are known to be common and long-persisting agricultural herbicides that are commonly used to destroy and prevent unwanted plant growth on farmland (Syafrudin et al., 2021). We use the following as our central research question: How well does *Pleurotus ostreatus*, the common oyster mushroom, degrade common long persisting groundwater pesticides?



Metolachlor



Acetochlor



Cyanazine

Because aligning our project with the One Health perspective is our foremost goal, it is important to consider the costs of pesticide use at the large scale it is presently used at. While pesticides may provide benefits such as increased crop yields, the aforementioned adverse effects imposed by these resources may do more harm than good when all facets of global health are considered. Reducing pesticide levels in both aquatic and terrestrial systems may reduce crop yields, but would also lead to better water quality and ecosystem health in farmlands. For pesticide use to align with the One Health ideology, the following minimum criteria would have to be met

in pesticide use; 1. Decreased pesticide chemical concentration in agricultural runoff, and 2. Decreased resistance in herbicide targeted species.

If this study concludes that pesticide uptake and degradation in culinary mushrooms is fairly high, we propose that further research be conducted on the subject so that culinary mushrooms may be cultivated with the intent of breaking down pesticides and minimizing adverse effects in ecosystems. Reducing pesticide levels in both aquatic and terrestrial systems may reduce crop yields, but would also lead to better water quality in farmlands and groundwater quality.

Section II

This study will focus on the effect of the herbicides acetochlor, cyanazine, and metolachlor on *Pleurotus ostreatus*, especially comparing how well *Pleurotus ostreatus* degrades the herbicides. The pesticides acetochlor, cyanazine, and metolachlor are long persistent herbicides that are used by agricultural companies for unwanted plant growth. Acetochlor, cyanazine, and metolachlor are most frequently found in streams and shallow groundwater. The study uses these herbicides because they are easily detectable in shallow water and there are not many studies comparing these herbicides, opposed to atrazine and others with a larger background of study (Purnomo, 2011).

P. ostreatus is classified as a white-rot fungi, which are environmental agents that assist in biodegradation and contribute to global carbon recycling. White-rot fungi can degrade endocrine disrupting chemicals, personal care products, and pharmaceuticals that cause toxicity to microorganisms. Mushrooms are crucial to study in the groundwater pollution scene because of their bioremediation capacity. Fungi has been researched to clean up contaminated sites from organic pollutants, petroleum, pharmaceuticals and pesticides. Fungi can also live in various climates and habitats, which allows for the fungi to be able to survive in treatment plants. Specifically, *Pleurotus ostreatus* has been studied to break down bleached kraft pulp mill effluent and polyaromatic hydrocarbons that grow in agro-industrial waste (Deshmukh et al., 2016). *Pleurotus ostreatus* has significant bioremediation properties. With a pesticide concentration of 8 mg/L with *Pleurotus ostreatus*, within 30 days, the pesticide concentration reduced to less than 4 mg/L (Karas et al., 2011).

One crucial property of *Pleurotus ostreatus,* is that it can degrade polyaromatic hydrocarbons (PAHs). PAHs are an environmental pollutant that can develop negative effects on humans during biotransformation, turning the PAHs into a carcinogenic substance. PAHs can arise in the environment from incomplete combustion, such as from industrial activities. Laccase and Mn-dependent peroxidase are the two main active enzymes in *Pleurotus ostreatus*. In a 41 day study, the enzyme activity of *P. ostreatus* varied over the time period. At least one of the enzymes was highly active at one time. These results signify that *Pleurotus ostreatus* continuously works in order to bioremediate PAHs (Baldrian et al., 2000).

Since *Pleurotus ostreatus* can degrade environmental pollutants, the objective of the proposed project will be to analyze how *Pleurotus ostreatus* degrades the long persisting pesticides acetochlor, cyanazine, and metolachlor. Pesticides affect the one health perspective by negatively impacting the environment, animals and humans. In agricultural cases, the listed herbicides are used to kill unwanted or surplus plants. When the pesticide is used, it is embedded in the soil, groundwater, and surface water, which allows for wide distribution. This distribution can help farmers and agricultural workers with their cause, however adversely impacts other one health parties. When the pesticides accumulate in the human cell membrane, it can interrupt the body's functions and cause hormone disruptions, immunosuppression, and chronic health problems (Purnomo, 2011).

Research previously done has shown the effects of pesticides and herbicides on humans and the distinct amount of the chemical that can be used to be deemed safe for humans and the environment. *Pleurotus ostreatus* has been studied extensively in the degradation of polyaromatic hydrocarbons from industrial externalities. However, there is minimal comparison of the *Pleurotus ostreatus* degradation to commonly used, long persisting pesticides. Our study will be on the groundwater contamination of the pesticides acetochlor, cyanazine, and metolachlor after the *Pleurotus ostreatus* degrades the pollutants in an incubator. We will analyze the *Pleurotus ostreatus* bioremediation of herbicides and compare the results of each type of herbicide to examine which herbicide *Pleurotus ostreatus* degrades best.

Section III

Preparation of fungus agar plates

Materials: 20 grams agar, 20 grams light malt extract, 1 L water, 40 petri dishes, Media bottle or jar with hole in lid, *P. ostreatus* inocula obtain from mushroom producere (How to Prepare Agar: Learn To Prepare Agar for Mushroom Cultivation, 2021)

Procedure (Purnomo et al., 2010):

1. Mixed 20 grams agar, 20 grams light malt extract well with 1000 mL water.
2. Pour dissolved solution into a sealed bottle and sterilize agar mixture in a high-pressure steam sterilizer at 120 celsius for 2 hours.
3. Place the bottle of hot agar mixture in the warm water bath to cool down agar.

4. Sterilize empty petri dishes and pour warm agar solution into petri dishes at aseptic operation stations. Pour approximately 20 mL of agar into each plate.

5. Ten grams of fungi mycelium inoculated into the petri dish, and then incubated at 22 celsius for 21 days adjust a moisture content of approximately 63% by water at the incubated room.

Quantitative Analysis

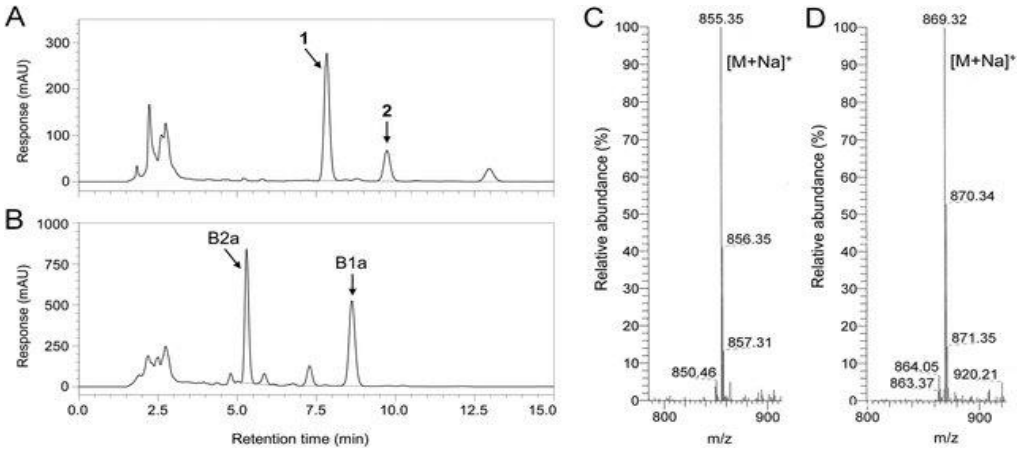
Spiking of the plates — To the control and colonized agar plates will be added the different pesticides at varying concentrations—0.50 μg/L, 1 μg/L, and 2 μg/L, or 0.5, 1, and 2 ppb, respectively. These concentrations reflect those detected in water across the United States, with cyanazine being found around 0.5 μg/L (World Health Organization, 2003), acetochlor at around 0.03 μg/L (Minnesota Department of Agriculture, 2020), and metolachlor around 0.03 μg/L (United States Environmental Protection Agency, 2014). The chosen test concentrations will account for natural degradates, which are often more abundant than the parent compound, as well as detection limits of the laboratory instruments. Three plates will be prepared per each pesticide/concentration combination to allow for statistical analysis of the data, giving a total of 48 plates. To each plate will be added 10 mL of each pesticide concentration evenly and the plates will be placed back into the incubator.

Sample analysis - The plates will be sampled a total of 5 times, once a week for five consecutive consecutive weeks, to establish a linear relationship amongst changes in concentrations. 1/10th of the plate will be removed via scalpel and prepared by crushing the sample and extracting with 1 mL dichloromethane (DCM). The DCM will be added to a small vial with the sample and stirred, being vacuum filtrated after. This will be repeated a total of 5 times, diluting back to 1 mL of DCM each time to account for solvent evaporation. The solution will then be transferred into a sample vial and run through LC-MS, with the peak area being recorded for each present peak per chromatogram, and the identity of each peak being checked against the mass spectra at the maximum of each peak. LC-MS combines high performance liquid chromatography (HPLC), which separates compounds by polarity, and Mass Spectroscopy (MS), which through ionization breaks the sample's molecules into smaller charged fragments which can be detected. The differences in these fragment peaks from the greatest peak give great insight into the structure of the analyte. LC-MS is particularly useful for this sort of analysis, as MS is run continuously with HPLC, so a peak corresponding to a different compound can be

identified as a specific compound. The mass spectrum of a peak can also be compared against a vast database of other compounds' spectra, allowing for easy computer identification.

Example of LC-MS data (Huang et al. 2015) *— HPLC data on the left, separate peaks indicating different compounds. Mass spec data on the right, C corresponding with peak 1, D with peak 2. The different peaks in the mass spec indicate different fragments.*

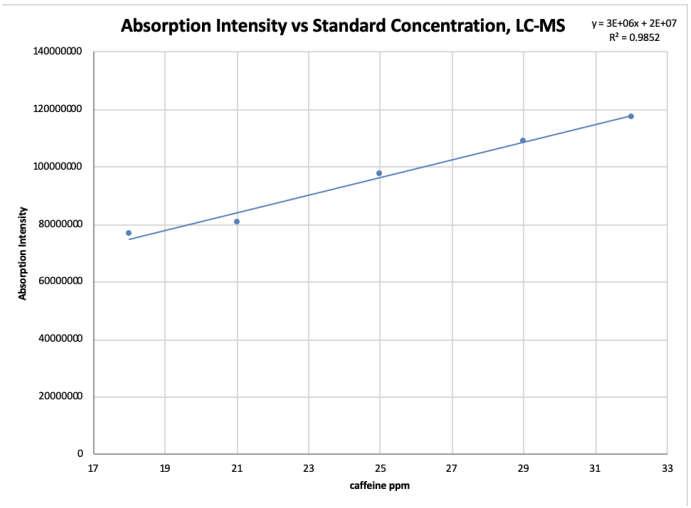
Development of a calibration curve — Given the microgram quantities per 10th of a plate and 10 decrease in solvent used for extraction, each sample should reflect the concentrations of the respective pesticide solution added, with a maximum of 0.5, 1, and 2 ppb for the 0.5, 1, and 2 ppb plates, respectively. A calibration curve will be developed by spiking a set of control plates with each pesticide using the same procedure for the experimental plates. Six points will be established for the calibration curves, with solutions of 0.25 ppb, 0.50 ppb, 1 ppb, 1.5 ppb, 2 ppb, and 3 ppb of each pesticide being made via dilution from pure stock. 10 mL of each pesticide solution will be added to the plates and allowed to sit for one day. The agar will be divided into 10 slivers, each extracted thoroughly with DCM. Each aliquot will be analyzed with LC-MS, and the peak areas will be recorded as if all the analyte had been extracted. This is

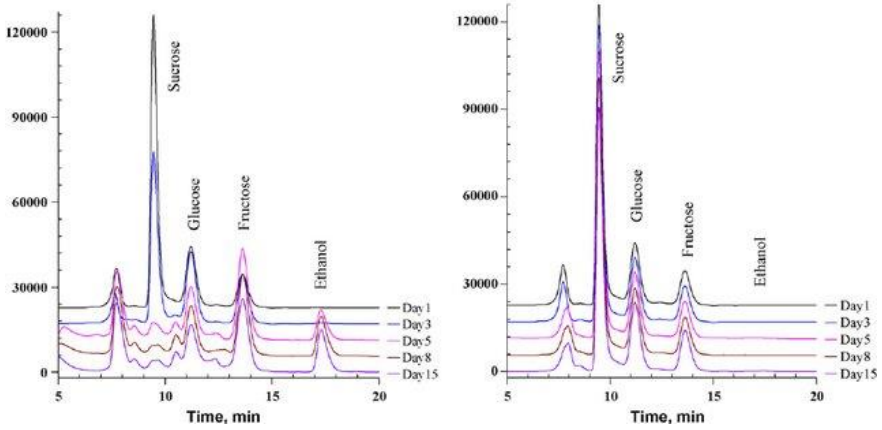


important because some analyte may be lost to the gel, but the fraction difference between concentrations in samples over time will be representative of the overall change in concentration. A plot of analyte signal intensity vs. standard concentration will be made and with the method of least squares, a line of best fit will be determined. This line of best fit will then be used to calculate ppb concentration of the pesticides over time. The graphic to the right indicates what a calibration curve could look like for LC-MS analysis. Standard addition could offer another useful method for quantifying concentrations of the products, but without knowing possible degradate identities, a series of response factors

cannot be developed prior to experimentation, rendering this technique useless despite the greater analysis it could provide.

Expected Results: A change in HPLC peaks is expected over time, indicating a time-dependent change in chemical composition. The chromatograms below (Wu et al., 2010) offer a great expectation of how the results should be. Initially, there should be one large peak for the original pesticide and other compounds present, but over time this should change, with each measurement. The chromatograms below compare sugar fermentation at room temperature (left) and refrigeration (right), so they do mirror how the results should come out, since the breakdown of pesticides by a fungus is a good analog to this. The change in peak area of the original pesticide peak over the course of the experiment will be a great indicator to the degradative capabilities of *Pleurotus ostreatus*.





Data and Statistical Analysis

Once the ppb concentration over time has been calculated for each of the three pesticides statistical tests will be used to compare the experimental values. An f-test will first be employed to examine the similarity between the variance of the values to evaluate precision of the data. These values will then be compared to one another using a t-test. This test will check for statistically significant average rates of change in ppb concentration. Statistical difference from the t-tests will

indicate a statistically significant change in concentration. This will indicate the efficacy of *Pleurotus ostreatus* bioremediation for each of the pesticides in this experiment.

Future Directions

This experiment only focuses on a single species of mushroom, but there are many other species and genera of fungi, including those that also see culinary use like *Pleurotus ostreatus* does. Conducting comparative studies using different species of mushroom may reveal an optimal subject for degrading pesticides. Other possible directions could look at how oyster mushrooms degrade different types of groundwater contaminants, or measure residual pesticide in mushrooms actively used for bioremediation to determine if they would be safe for human consumption. This latter idea has strong implications for an intervention in line with the One Health perspective, as while bioremediating mushrooms directly benefit environmental health, they may also have effects on human health.

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