Mycoremediation Project: Using Mycelium to Clean Up Diesel-Contaminated Soil in Orleans, California

Levon Durr[1]
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Background:

Mycoremediation, or fungal bioremediation, is the process of using fungi to improve the ecological health of a site by either degrading contaminants or sequestering them for removal.

The use of fungi and bacteria for the degradation of organic compounds has been employed since the mid-twentieth century.[2] The ability of the hyphae, the root like structure of the fungus, to molecularly disassemble organic compounds can be applied to environmental toxic cleanup. Mycelium, which is a mass of vegetative hyphae, secretes digestive enzymes that have the ability to break hydrocarbon bonds into smaller less toxic molecules. The mycelium can essentially turn the hydrocarbons into carbohydrates.

Oyster mushroom, or *Pleurotos ostreatus*, is a white rot fungus that produces digestive enzymes such as lignin peroxidase, manganese peroxidases and laccases to break down wood.[3] Wood and petroleum-based fuels contain similar hydrocarbons, making white rot fungus a great tool for mycoremediation. Testing and laboratory results of the ability of fungi to degrade toxins are now well documented. Emerging research has demonstrated how these technologies can be applied in the field. Each site's microclimate brings new challenges and new lessons that help establish techniques for generations to come.

In the spring of 2011 Mark DuPont, then-President of the Mid Klamath Watershed Counsel (MKWC), contacted us at Fungaia Farm regarding a diesel fuel spill behind the Panamnik Building in Orleans, California. The Panamnik Building houses the MKWC offices, as well as the Orleans Community Center.

Fig. 1. The contaminated site being excavated

The contaminated site previously had a backup generator and an above ground fuel storage tank. The generator had leaked motor oil onto the ground and the fuel storage tank had leaked diesel fuel into the surrounding soil (Fig. 1). The site was about 60 yards away from the Klamath River and the soil consisted of sandy loam. MKWK was interested in hiring Fungaia Farm to implement the use of fungus to break down the contaminants in the soil and remediate the site.

At Fungaia Farm, located just outside of Eureka, California, our mission is to provide mushroom spawn to home and commercial growers for food production and for environmental cleanup

[1] Owner of Fungaia Farm. For more information contact fungaiafarm@gmail.com.
[2] Mycoremediation: Fungal Bioremediation, Singh, 2006
[3] Schliephake et al., 2003

through mycoremediation. After assessing the site, Fungaia Farm decided to take on this project, since it was exactly the type of project we were looking for.

Together with Michael Stearns, Panamnik Building Coordinator, and Mark DuPont, we came up with a plan to address the contamination. Based on this plan, MKWC received a Brownfields Grant through Humboldt County for soil remediation.[4] The plan was to remediate the contaminated site and subsequently build an outdoor stage for community events.

Methods:

We started the project that fall, once the weather was cool enough for the mycelium to survive. In late September we began expanding oyster mushroom grain spawn to start our first inoculation of the soil that was to be removed from the contaminated site. We chose a commercial oyster strain, *Pleurotus ostreatus*, because it can thrive in wide temperature variations and is aggressive at breaking down hydrocarbons (Fig. 2).

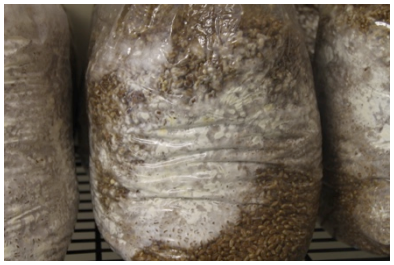

Fig. 2. Grain spawn: wheat berries inoculated with oyster mycelium

On October 7, 2011 we began the process of pasteurizing enough straw to fill 150 20-inch burlap sacks. We inoculated the pasteurized straw with the grain spawn as we hand-stuffed it into the burlap sacks. These inoculated burlap sacks were allowed to colonize for ten days (Fig. 3). At the peak of their growth we transported them to the remediation site in Orleans. When we arrived at the project site, MKWC began removal of the contaminated soil, overseen by LACO Associates, a local engineering firm, and Ron Reed, a Karuk tribal officer.

Fig. 3. The process of creating inoculated burlap sacks: first the straw and sacks are pasteurized using 180° F water, then the sacks are stuffed with straw and grain spawn. After approximately 10 days, the white mycelium had thoroughly colonized the sacks.

We set up our first test plot by building a rectangle of straw bales covered with black plastic in order to contain the contaminated soil. We then layered the inoculated burlap sacks with cardboard and contaminated soil (Fig. 4). The cardboard provided an extra food source for the mycelium. The piles were kept covered in plastic per the county's request. We treated

[4] United States Environmental Protection Agency (USEPA) Brownfield Cleanup Revolving Loan Fund managed by the Humboldt County Economic Development Department

approximately five to seven yards of soil using this method. By November 12 we began to see signs of mycelial growth from the burlap sacks into the contaminated soil. Though we saw mycelial growth, problems arose during the winter rains when the plastic covering the pile came loose and allowed rain water to drown some of the mycelium. Working with this more traditional containment method created major challenges in controlling the moisture levels. This pile would eventually be added to the final six piles and receive a second treatment.

Fig. 4. Inoculated burlap sacks being laid out and layered with contaminated soil

As it turned out, there was more contaminated soil than was initially estimated. This sent us back to the drawing board to design a less labor-intensive method for growing and transporting a sufficient amount of mycelium to the site, as we now had 40 to 60 yards of contaminated soil needing treatment. Meanwhile, the contaminated soil was wrapped in black plastic and placed nearby to avoid any further leaching of hydrocarbons.

In order to reduce labor costs, we decided to remove pasteurized straw from the equation and inoculate large rolls of burlap directly with the grain spawn (Fig. 5). The inoculated burlap would then be layered with fresh straw and contaminated soil. We ran some small tests at our facility, layering inoculated burlap with wet fresh straw and soil contaminated with unleaded gasoline. We measured a 90% reduction in hydrocarbons after two months. After the success of our experiment we began gathering the necessary materials to treat approximately half of the contaminated soil.

Eleven 300-yard rolls of hydrocarbon-free burlap were pasteurized in 180-degree water for one hour, and then inoculated with oyster grain spawn. The inoculated burlap rolls were wrapped in plastic and allowed to colonize for two weeks.

Fig. 5. Inoculating rolls of burlap with oyster grain spawn; the colonized rolls after two weeks

On May 24, 2012 we transported the inoculated burlap rolls to the job site. Michael Stearns had a large truckload of rice straw delivered to the site. A crew of six people and a bobcat driver spent

the next six hours applying a layer of straw, then a layer of inoculated burlap, then another layer of straw, followed by a layer of contaminated soil (Fig. 6). The straw layers were moistened as they were applied. We repeated this layering procedure in two piles that were each 20 feet long, six feet wide, and five feet high. We treated approximately half of the contaminated soil at this point, approximately 20 to 30 yards.

Fig. 6. Layering straw, inoculated burlap, and contaminated soil into piles

The piles were located next to the parking area in full sun. Black plastic was laid down under the piles and wrapped over the tops once the piles were completed. The piles showed healthy mycelial growth throughout May and June, but by July, temperatures in the piles had risen above optimal range and most of the growth had ceased. The plastic was removed and the piles were watered periodically throughout the summer. Some new mycelial growth was detected during the following fall and spring. The soil also began to show other signs of life in the forms of red worms, toads and other fungi (Fig. 7).

Fig. 7. Contaminated soil piles showing signs of life: healthy oyster mushroom mycelium, worm castings and other native fungi

On April 27, 2013, because the piles exceeded optimal temperature and had slow mycelial growth, we decided to apply a second treatment to the previously treated soil. All piles were moved into the shade to reduce internal temperatures, including the original pile treated with

the burlap sacks. We used the same method for this second treatment: inoculated burlap rolls layered with straw.

Four burlap rolls were used with the addition of 25 bags of spent Oyster spawn from Mycality Mushrooms in Arcata, California. The bags were approximately ten inches wide by six feet long and filled with pasteurized straw that had been used to grow Oyster mushrooms. This spent substrate was used as a layer on the bottom of each of three of piles during this second treatment (Fig. 8).



Fig. 8. Relocating treated soil piles to shady location, re-treating with four inoculated burlap rolls and applying spent oyster spawn from Mycality Mushrooms to the bottom of the new piles

On May 29, 2013, we applied the final treatment to the rest of the untreated contaminated soil (Fig. 9). As mentioned before, this soil had been kept wrapped in black plastic to avoid any further leaching of the diesel fuel or motor oil into the surrounding environment. Approximately 20 to 30 yards of soil still needed treatment. We implemented the same methods using the inoculated burlap rolls layered in between straw and contaminated soil. Three more piles were built in the shade next to the three re-treated piles. Ten 300-yard rolls of inoculated burlap were used in this final treatment.



Fig. 9. Final treatment of remaining untreated contaminated soil

Temperatures were taken of the piles constructed in April. Internal temperatures ranged from 65-75 degrees, which was on target for optimal mycelial growth. Moving the piles in the shade and keeping the piles shorter than four feet high seemed to help keep internal temperatures lower.

Assuming most of the biological activity in the pile would be during the milder months of spring and fall, our plan was to let the mycelium and bacteria have a chance to go through a full year cycle, to allow the break down of hydrocarbons when temperatures were above 50 degrees and below 75. Temperature is a major factor for healthy mycelial growth during a remediation project. Milder climates offer a much bigger window for biological activity and allow more leeway in size and placement of the substrate being remediated. Orleans is located in the northeast corner of Humboldt County, California, and has an average summer temperature in the mid-90's and winter temperatures in the mid-30's. This meant that we had to not only do the inoculation of the contaminated soil during the milder times of year, but also gave us less options for the size and placement of the piles and a smaller window for optimal growth.

In late May, once all the soil was inoculated, we monitored the piles as mycelium spread from the burlap onto the straw and into the contaminated soil (Fig. 10). Healthy mycelium was seen colonizing the piles through the month of June and then began to slow down as the weather warmed and the straw began to break down. Other straw-loving fungi also began colonizing and fruiting on the straw.

Fig. 10. Piles reconstructed in April showing healthy mycelium and optimal temperature for mycelial growth

Drip tape was set up to keep the piles moist through the summer months. The piles were covered in black plastic, but were uncovered periodically to allow the piles to breathe. Even with the piles in the shade, some still exceeded optimal temperatures. This problem was most likely because the pile size generated too much internal heat from biological activity and because the ambient outside temperatures were rising.

The piles reduced dramatically in mass as the summer passed and the straw and burlap fully decomposed. By late fall red worms had moved in and left thick layers of worm casting on the surface as well as within the piles. Test holes dug into the piles produced no signs of diesel fuel odor.


Fig. 11. Remediated piles showing healthy sign of red worm activity

Prior to the final soil testing scheduled for the spring of 2014, we decided to inoculate the pile with compost tea to increase the biological activity in the treated soil. We also hoped to further reduce any contaminants and introduce a healthy blend of organisms into the soil that had been contaminated and wrapped in plastic for two years. The compost tea was made using fish hydrolysate, compost, and dried oyster mushrooms. Damian Pack, a resident of Orleans, brewed the tea in spring water overnight in aerated tanks.

Hoping to allow enough time for the bacteria to colonize the piles before the final soil testing, we applied the compost tea early in the spring of 2014. Outside temperatures were below optimal but the hope was that as the ground warmed into spring, the bacteria would become active in the soil.

With snow still on the ground, we used a compost auger and drilled holes into the tops of the piles every few feet to facilitate penetration into the piles. The tea was pumped out of the brewing tank with a diaphragm water pump to avoid any damage to the bacteria during the pumping process. The tea was spread over three of the piles with augured holes and applied just to the tops of the remaining three piles. The piles were then re-covered with black plastic and allowed to rest.


Fig. 12. Drilling holes into pile with auger


Fig. 13. Compost tea tank with diaphragm pump; compost tea being applied to augured holes in piles

On June 6, 2014 LACO collected samples from all of the piles and sent them to KIFF Analytical in Davis, California. As described in the results section below, they confirmed that remedial target concentrations for diesel and motor oil had been reached.

Results:

Pre-remediation soil samples collected by LACO Associates and analyzed at KIFF Analytical indicated diesel fuel concentrations as high as 250 mg/kg and motor oil concentrations as high as 6,200 mg/kg in the site's soils.[5] The goal for the project was to reduce these levels to below 50 mg/kg of diesel and below 370 mg/kg of motor oil, levels which would fall within acceptable limits set by the state for these contaminants.[6] A representative composite sample tested by KIFF Analytical at the end of the project showed concentrations of 49 mg/kg of diesel and 47 mg/kg of motor oil, both below target levels.[7]

In an effort to draw more precise conclusions, in conjunction with MKWC, we sent samples to North Coast Laboratories to test soil samples that had received slightly different treatments. Some samples had been treated once with mycelium-inoculated material and some had been treated twice (see Methods for details). A sample of contaminated soil had been taken from the project site prior to any treatment and had been stored in a sealed, airtight container for the duration of the project. The results of this pre-treatment soil sample were then compared with the results of the variously treated samples (Fig. 14 and 15).

[5] Testing showed that the contamination was variable across the site, with some samples showing relatively low concentrations in the range of the target remediation levels, while others showed concentrations two orders of magnitude higher. Unfortunately, the high concentrations of diesel in some samples could not be precisely quantified, due to samples being outside of control limits, though all parties recognized that contamination levels were extremely high in some areas.

[6] Information on initial field testing is summarized from "Remedial Action Plan for Former Generator Shed," prepared for Mid-Klamath Watershed Council by LACO Associates and dated August 24, 2011.

[7] Information on soil sample analysis and results is from "Final Report – Panamnik Building – Former Generator Fuel/Oil Spill Cleanup," prepared for Mid-Klamath Watershed Council by LACO Associates, July 25, 2014.

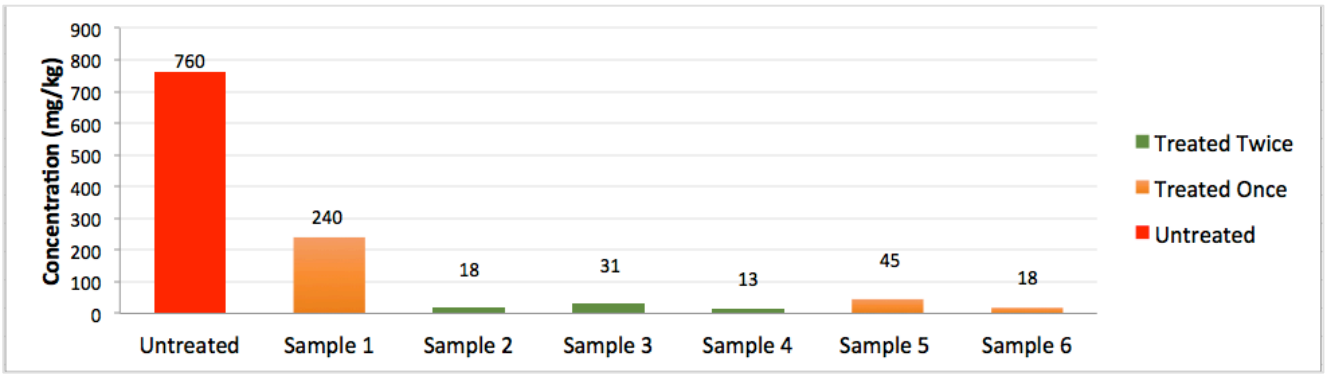

Fig. 14. Diesel concentrations of treated and untreated soil as measured by North Coast Laboratories

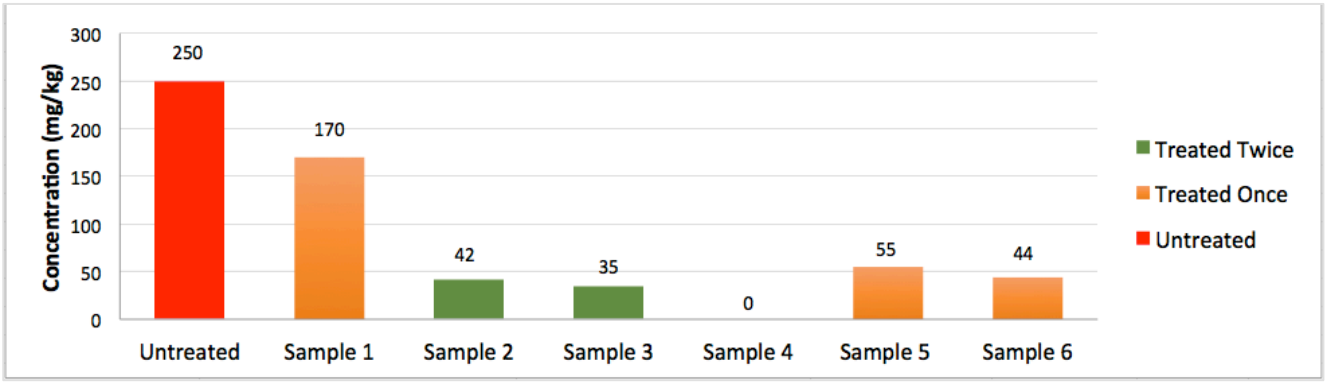

Fig. 15. Motor oil concentrations of treated and untreated soil as measured by North Coast Laboratories

The untreated soil sample tested by North Coast Laboratories showed diesel concentrations of 760 mg/kg and motor oil concentrations of 250 mg/kg.[8] Five of the six-treated samples had levels of contamination ranging from 18 to 45 mg/kg diesel and from undetectable to 55 mg/kg motor oil. The remaining sample had substantially higher levels, with 240 mg/kg diesel and 170 mg/kg motor oil. Though sample 1 did not meet the target reduction to 50 mg/kg of diesel, the other five samples met the target for diesel and all of the samples met the target levels for motor oil. Further, all of the samples indicate a substantial reduction from baseline levels.

Discussion:

The project was successful; the remedial targets were met, demonstrating the effectiveness of mycoremediation for environmental cleanup of soil contaminated with diesel fuel and motor oil. Further, remediation of the soil on site avoided costly soil disposal and addressed the problem directly rather than merely shipping it to a landfill in a different watershed. Since the site was culturally significant for the Karuk Tribe, minimizing soil disturbance and removal was another advantage. Following the remediation, MKWC was able to use the soil as landscaping material at the site. Additionally, by conducting the project on site, the community and other interested parties were able to participate in the project and learn about the remediation process.

[8] It is unclear whether these figures should be directly compared to those analyzed at KIFF Analytical. The same test method (EPA 8015) was used; however, KIFF Analytical conducted an additional pre-treatment step using silica gel in some of their analyses and North Coast Laboratories references EPA 3550 along with EPA 8015 in their analytical methods.

Implementing mycoremediation in the field often presents challenges due to unexpected circumstances, weather conditions, and site-specific requirements. This project was no exception and provided learning experiences for all involved. Some key insights from this project and new ideas for future projects are described below.

Additional Testing

To better understand the effect of the fungi on the hydrocarbons and at what point the remediation occurred, more testing should be done throughout the process. Even though most of the piles that were treated twice showed lower levels of hydrocarbons than the piles that were treated once, periodic soil tests conducted throughout the process would provide additional information to better understand the progression of the remediation. Without interim tests, the effectiveness of each individual treatment is unknown.

Additionally, during the initial soil excavation, testing showed the concentration of hydrocarbons was much higher at ground level than at the bottom of the excavation site. However, during the creation of the piles, all of the soil was mixed, and there was no record of the initial concentration of hydrocarbons in each pile. Tracking the more heavily contaminated soil throughout the remediation process would not only provide greater insight into the impact of the remediation, but would also enable us to treat the more highly contaminated soil more aggressively, such as with additional mycelium or repeated treatments.

In future projects, testing and tracking of different samples under different treatment regimes should be a top priority. Nevertheless, *all* treated samples showed substantially lower contamination levels than the untreated sample, demonstrating the effectiveness of the remediation.

Mycelial Production

The health of the mycelium is paramount to the success of the project. Initially, and throughout the entire project, the monitoring and needs of the fungus must be a top priority.

We feel that the use of burlap rolls to transport the mycelium to the site offers a significant innovation in the field of mycoremediation. However, in the process of inoculating the burlap rolls we learned the importance of temperature monitoring. Biological activity during colonization of some of the rolls caused them to overheat, causing the mycelium to collapse and allowing yeast or molds to take over. In this project we used 300-yard rolls of burlap, but recommend using 100 or 150-yard rolls in the future. Ideally, the inoculated rolls should not exceed approximately 10 inches in diameter. Smaller rolls would prevent overheating during incubation and make the rolls more manageable for transport and handling.

Contamination of the inculcated burlap rolls might also be mitigated by the use of sawdust or straw spawn, rather than grain spawn, as an inoculant. Sawdust or straw, being less nutritious food sources, are less likely to feed yeasts, molds and other potential contaminants. Additionally, because grain is expensive, expanding the grain spawn to sawdust or straw could significantly reduce costs associated with spawn production.

Size and Placement of Piles

We quickly learned that the size and placement of the inoculated piles dramatically affects the health of the mycelium. During the initial excavation, an unexpectedly large amount of soil was removed. In an effort to fit this soil into the designated area for building the piles, we built piles

that were much too large. Further, these over-sized piles were placed in the full sun. Both factors caused temperatures to exceed the optimal range for the mycelium. After moving the project into the shade and ensuring piles were no more than four feet high and three feet wide, the mycelial growth improved.

Though the three-foot by four-foot piles functioned adequately in this project, we noted that the internal temperature of the piles often rose higher than desired. We believe even smaller piles (2 ft. x 2 ft.) would be ideal to control temperatures, particularly for remediation projects in warmer climates.

Layering of Substrate

As expected, sandwiching the inoculated burlap between rice straw gave the mycelium an instant food source and allowed for some of the contaminated soil to be integrated into the straw as it was layered onto the piles. We could then see mycelium growing off the burlap onto the straw and running into the contaminated soil. The straw fibers became pathways for the mycelium to travel as it impregnated the contaminated soil.

Equally important as layering with straw, is ensuring the proper ratio of contaminated soil to straw and inoculated burlap. For the mycelium to come in contact with the hydrocarbons, we attempted to keep the soil layers between three to five inches. However, the depth was hard to control with heavy machinery running and five people working. Areas that exceeded the optimal three to five inches of soil often appeared to be less colonized.

Moisture Levels

Moisture levels are also extremely important for healthy mycelial growth. In Northern California's Mediterranean climate with hot summers and rainy winters, we had to take several measures to maintain proper moisture in the piles. A thick layer of straw was used to cover the piles during remediation to mitigate moisture loss and keep the piles cool. Black plastic was kept over the piles during periods of rain to avoid oversaturation and stop any leaching of the hydrocarbons into the surrounding soil. Periodic watering of the piles during the dry summer months helped to offset the moisture loss from evaporation.

Another challenge we faced early on was the unexpected requirement to fully contain the contaminated soil in plastic during the remediation process. In our first attempt to accommodate this requirement, we built a containment out of straw bales lined with plastic. This became problematic when the plastic cover failed to keep rain out, did not permit the piles to dry, and drowned the mycelium. In subsequent treatments, we used a different method of wrapping the piles in plastic that enabled better moisture management. Determining relevant regulatory guidelines in advance of beginning the project will help avoid some of these issues. Additionally, we recommend working with regulatory authorities to take into account the needs of the remediation project to ensure that regulations allow for proper moisture and airflow for the mycelium. Mycoremediation-friendly regulations can become crucial factors for a successful outcome.

Additional Treatments

As described earlier, throughout the three years of the project, multiple treatments were employed to reduce the level of diesel in the soil. Without knowing the initial concentration of contaminants in each pile, one cannot make absolute conclusions; however, the data suggests that the piles that received two treatments of mycelium had lower levels of contaminants.

Additionally, in the last year of the project, we treated the soil with a biologically active compost tea. Unfortunately, there were no distinct differences in the concentrations between piles that had been treated with the tea and those that had not. Though we do not have strong proof of the efficacy of multiple treatments, we still recommend treating the soil more than once and considering alternative biological treatments that can be used in conjunction with mycoremediation.

Acknowledgments:

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