Remediation of pesticide contaminated soils: alteration of pH by *Trametes spp*. fungi?

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ABSTRACT

Pesticide drift from organophosphate insecticides near industrial farms is a big problem in Costa Rica. Excess organophosphates run into waterways and nearby soils, where they bioaccumulate in the foods grown there and in the drinking water, causing health problems to those who consume them. Some mushrooms have bioremedial effects on pesticides. *Trametes versicolor*, a species of white rot fungi, can break down organophosphates by secreting chemicals that catalyze degradation reactions in soils. To determine if this is a viable option for bioremediation in the tropics, I observed four *Trametes spp*. fruiting bodies and one substrate without fungus after adding Malathion organophosphate pesticide. I observed pH, organophosphate content, and insect presence and species richness over a period of 11 days to quantify the mushroom's effects on the pesticide. On the substrates with mushrooms, pH was significantly higher than the substrate without. This is beneficial because high pH promotes hydrolysis of organophosphates, which is the process by which these pesticides are most successfully broken down and removed from the environment. Substrates with mushrooms also had greater insect presence and insect species richness than the substrate without, even after pesticide application. Thus, I hypothesize that *Trametes spp*. fungi are altering soil pH and creating an environment conducive to organophosphate breakdown, which means they could be viable options for bioremediation.

Remediación de Suelos Contaminados por Pesticidas: ¿Alteración de pH por los Hongos *Trametes spp*.?

RESUMEN

La contaminación por insecticidas organofosforados cerca de fincas industriales es un gran problema en Costa Rica. El exceso de organofosforados corre en los cuerpos de agua y suelos cercanos, donde se bioacumulan en los alimentos producidos y en el agua potable, causando problemas de salud a quienes los consumen. Algunos hongos tienen efectos de bio-remediación sobre pesticidas. *Trametes versicolor*, una especie de hongo que degrada lignina, puede descomponer organofosfatos secretando sustancias químicas que catalizan reacciones de degradación en suelos. Para determinar si es una opción viable para la bio-remediación en los trópicos, estudié el sustrato bajo cuatro cuerpos fructíferos de *Trametes spp.*, y un sustrato sin hongo, antes y después de añadir el pesticida Malathion organofosfato. Estudié el pH, el contenido de organofosfato y la riqueza de especies de insectos durante un período de 11 días

para cuantificar los efectos del hongo sobre el pesticida. En los sustratos con hongos, el pH fue significativamente mayor que en el sustrato sin hongo. Esto es beneficioso porque un pH alto promueve la hidrólisis de los organofosfatos, proceso por el cual estos pesticidas se descomponen con mayor éxito y son eliminados del ambiente. Los sustratos con hongos también presentaron mayor abundancia y riqueza de especies de insectos que el sustrato sin hongos, incluso después de la aplicación de pesticidas. Por lo tanto, propongo la hipótesis que los hongos *Trametes spp*. están alterando el pH del suelo y creando un ambiente propicio para la descomposición de organofosforados, lo que significa que podrían ser opciones viables para la bio-remediación.

The industrialization of agriculture and use of monocropping is responsible for countless pest epidemics, many of which are combatted with the use of pesticides. As the world becomes more reliant on industrial crops to feed our growing human population, we are faced with the problem of pollution from our excessive use of pesticide. In Costa Rica, large corporations have planted extensive monocultures of pineapple and banana that use chemicals such as organophosphates and other pesticides, causing the country to be one of the largest per capita consumers of agrochemicals in the world (Araya *et al*. 2014). In fact, Costa Rican consumption of agrochemicals is, on average, 18.2 kilograms of chemicals per hectare of cropland (Regional Institute for Studies in Toxic Substances 2015). These agrochemicals seep into waterways and drift into nearby soils, becoming an unbelievable health risk for surrounding communities (Wesseling *et al*. 1993). Organophosphates are particularly dangerous because they contain the same active compounds as nerve gases— compounds that cause serious central nervous system depression, seizures, infertility, and in some cases, even death (Bardin *et al*. 1994). Thus, finding solutions to pesticide drift is extremely important for ensuring that underprivileged communities near industrial farms have access to clean soil and water so they can grow food and drink clean water without facing serious health problems.

Though organophosphates are not known to be especially persistent in soils, they can easily bioaccumulate, as they are extremely soluble in organic compounds such as fruits and animal fats (Davies *et al*. 1975). This is why contaminated soils pose such a threat to humans — fruits and vegetables grown in these soils will contain high pesticide content and, when consumed, will result in the absorption of the chemical into body fats.

In many cases, mushrooms have been used as a form of natural and cheap soil bioremediation. White rot fungi is a broad category of many species of Basidiomycetes that grow on wood and are characterized by the white color the wood turns as the fungi break down the lignin. This category of fungi excretes peroxidases into its substrates that act as catalysts to the oxidation and reduction reactions that degrade large organic molecules and environmental pollutants (Aust 1995). The ability to facilitate degradation of chemicals in soils could suggest that these fungi may be useful bioremediators of chemical pollutants such as pesticides. In particular, the white rot species *Trametes versicolor* has been found to catalyze the breakdown of various nerve gases and toxins like organophosphates by excreting facilitative chemicals into its substrate (Stamets 2005). When broken down as such, organophosphates separate into inert phosphorus esters and alcohols and are no longer an environmental health threat.

Since organophosphates are some of the most used pesticides in Costa Rica today (Araya et. al 2014), *Trametes versicolor* could potentially be a viable option to help bioremediate the soils of communities in the path of pesticide drift from industrial farms. Since *Trametes versicolor* may be difficult to find in some regions in Costa Rica, my study included a variety of *Trametes spp.* fungi. Based on this, I posed the question: can *Trametes spp.* fungi aid in the bioremediation of soils contaminated by organophosphate pesticides?

MATERIALS AND METHODS

To test the bioremedial properties of *Trametes spp.* mushrooms, I applied Malathion pesticide (an organophosphate pesticide composed of 96% inactive ingredient and 4% active O,O-dimethyl dithiophosphate) to four different substrates: the substrate under the fruiting body of three *Trametes spp.* fungi and a substrate without a visible mushroom fruiting body (Figure A). I then observed and compared a *Trametes spp.* mushroom without pesticide. For 11 days, I measured pH, organophosphate content, and insect presence on the study sites located in the forest around the Monteverde Institute (Figure A).

To start my procedure, I identified four relatively undisturbed *Trametes spp.* fruiting bodies using the "Totally True Turkey Tail Test" identification sheet from MushroomExpert.com. I ensured that *Trametes spp.* was the only visible fungi in each plot. I then identified one clear patch of substrate that had no visible evidence of *Trametes spp.* fungus or mycelia and confirmed this by ensuring that the substrate wood was not the characteristically white color it would turn if there was presence of white rot fungi. I found all fungi on decaying logs near the forest floor and at least 10 meters apart from each other (Figure 1). Around all four fruiting bodies and the patch of substrate, I marked a 35 centimeter by 35 centimeter square with flagging tape. Though I did not start sampling the *Trametes spp.* without pesticide (Control Mushroom) until the fifth day of the experiment, I took samples of the substrate from each other plot (Mushrooms 1-3 and No Mushroom) before applying pesticide. To take a sample, I used a spoon to scoop wood from the log beneath and around the fungus fruiting body. The day of the first sample, without pesticide, is marked as "Day 0" in all figures and tables. The next day ("Day 1"), I made a Malathion dilution based on the manufacturer's recommended recipe (one part Malathion to 384 parts water) using 0.52 grams of Malathion and 200 milliliters of distilled water. I applied the Malathion insecticide dilution in equal amounts (five sprays from a laboratory spray bottle per fungus specimen) on the substrate of the four plots and left one plot pesticide free (Figure 1). I then took samples of the wood substrate from each plot immediately after applying the insecticide, using the same sample methodology as previously listed. I continued to return to the plots and take samples from each site for 9 consecutive days after the pesticide application.

Each day, I performed a set of tests on each sample. With each sample, I crushed the pieces of wood and soil as much as possible and then mixed one part crushed sample substrate with one part distilled water. I then used a pH meter to test pH of each individual sample. With this substrate and water mixture, I used a Canon Organophosphate Test Kit to determine parts per million of organophosphate by performing a titration and following the instructions in the kit. I translated each drop of titrant used until color change to 0.7 parts per million of active organophosphate content in my solution, as described in the test kit. I then compared the results of pH and organophosphate content between each plot over time. For the pH results over time I

conducted a Kolmogorov Smirnov test. For the average pH for all plots I conducted an ANOVA to test for significant differences.

To test the accuracy of my organophosphate test kit, I created a dilution with 20 milliliters of my previously mixed Malathion dilution with 60 milliliters of distilled water. I calculated the parts per million active organophosphate content by using mass and percentage of active chemical listed on the Malathion packaging. I then tested the solution's pH and performed a titration according to the instructions in the test kit and translated each drop of titrant into parts per million active organophosphate.

At the time I took each substrate sample, I also took a survey of insects within each plot to see if insects might respond to the pesticide or serve as an indicator of pesticide degradation. I used two measures to survey insects: insect presence and insect species richness. I defined insect presence as the number of visible insects on each plot at any one time and created a scale from "no insect presence" to "high insect presence." "No presence" was marked by the absence of visible insects from the substrate. "Low presence" indicated one to two visible insects, "medium presence" indicated two to five visible insects, and "high presence" indicated more than five visible insects at time of sample collection. The second measure, "insect species richness," was defined as the number of morphospecies of visible insects present on the substrate. The scale, again, ranged from "no" to "high." "No species richness" indicated that no visible insects were present, "low richness" that only one morphospecies of insect was present, "medium richness" that two to four morphospecies were present or there were many morphospecies with a clear dominance of one, and "high richness" indicated that there were five or more morphospecies or that each visible insect was of a different morphospecies.

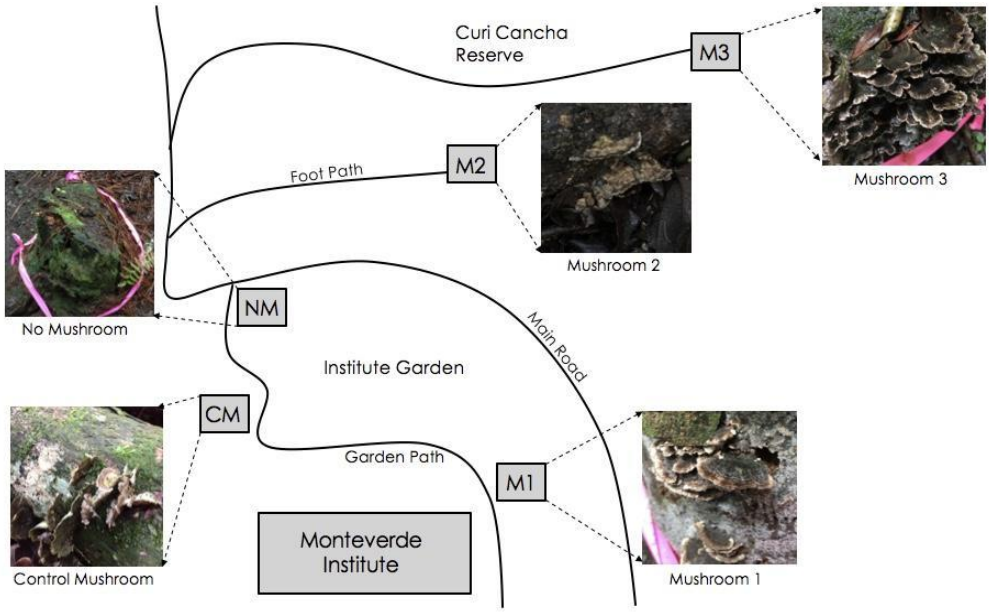

Figure 1. Map of experimental plots around the Monteverde Institute in Monteverde, Puntarenas, Costa Rica. Each plot represents a 35 cm by 35 cm square and consisted of a decaying log with *Trametes spp.* fungi. Malathion pesticide was applied to the No mushroom, Mushroom 1, Mushroom 2, and Mushroom 3 plots, while Control mushroom remained free of pesticide for the entire length of the experiment. I took substrate samples daily from each of these plots.

RESULTS

The mushroom substrates studied had significantly higher pH than the substrates without mushrooms, both before and after pesticide application (Figures 2 and 3. $F_{(4,45)} = 11.87$, $p<0.0001$). Of the mushrooms, Mushroom 2 exhibited the highest average pH. I also observed that organophosphate content did not exhibit expected behavior (Figure 4), and I ran a test for organophosphate kit accuracy (Table 2). The accuracy test seemed to show that the kit exhibited some inaccuracies in registering pesticide content. Insect presence and species richness observations showed that mushroom substrates had both higher insect presence and species richness than the no mushroom substrate (Figures 5 and 6). Of all the mushrooms, Mushroom 1 exhibited the highest insect presence and species richness. The No Mushroom plot had the lowest pH and insect presence and species richness of all sample plots. Some mushrooms showed signs of aging (Mushroom 1, Mushroom 3); the fruiting bodies had a light coating of moss. It rained everyday over the course of this experiment, starting off relatively wet for the first 6 days, and ending with an abrupt decrease in rain for the second half of the experiment.

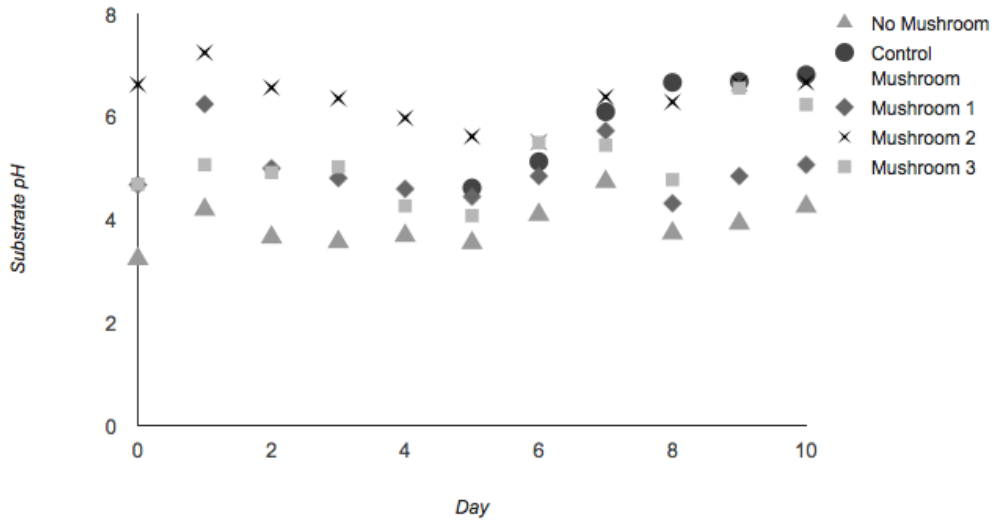

Figure 2. The pH values of substrate samples over time (in days). Day 1 shows the addition of pesticide. Each curve remains relatively constant, with a slight dip and increase around the sixth day, and with mushroom substrates showing higher pH than no mushroom, even on Day 0 before pesticides were added. The difference in pH over time shows a significant difference between each mushroom and the no mushroom substrate. See Table 1 for statistical comparisons.

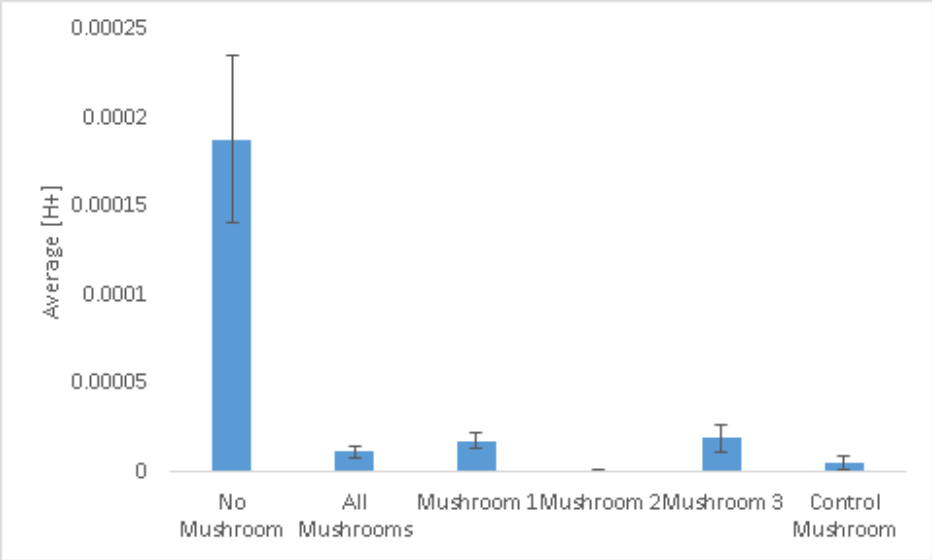

Figure 3: Average [H+] concentration in both the mushroom and no mushroom plots and includes an average for all mushroom plots together (All Mushroom). High [H+] indicates greater acidity and lower pH. There are significant differences between the average [H+] of the no mushroom plot and the plots with mushrooms ($F_{(4,45)} = 11.87$, $p<0.0001$).

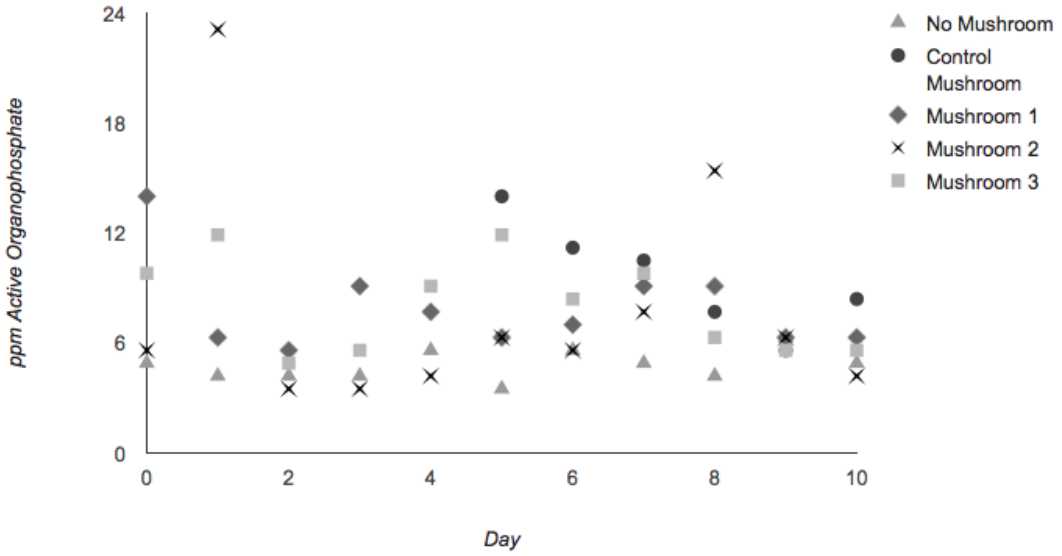

Figure 4. Parts per million active organophosphate content in substrates from each plot over time. Data is variable and does not show expected results, registering a relatively high organophosphate content on Day 0 before application and then, on Day 1, in all plots except Mushroom 2 and 3, registering lower content after pesticide application. See Table 2 for results of accuracy test of kit.

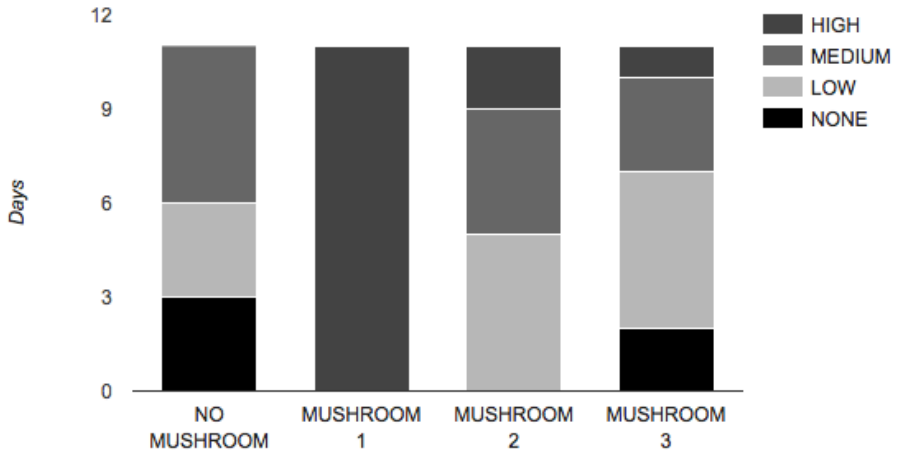

Figure 5. Number of days that each study site showed None, Low, Medium, or High insect presence. Overall, plots with mushrooms tended to have higher insect presence than the plot without a mushroom. Control Mushroom is omitted due to lack of insect presence data. For full Control Mushroom insect presence data, see Appendices 5 and 7.

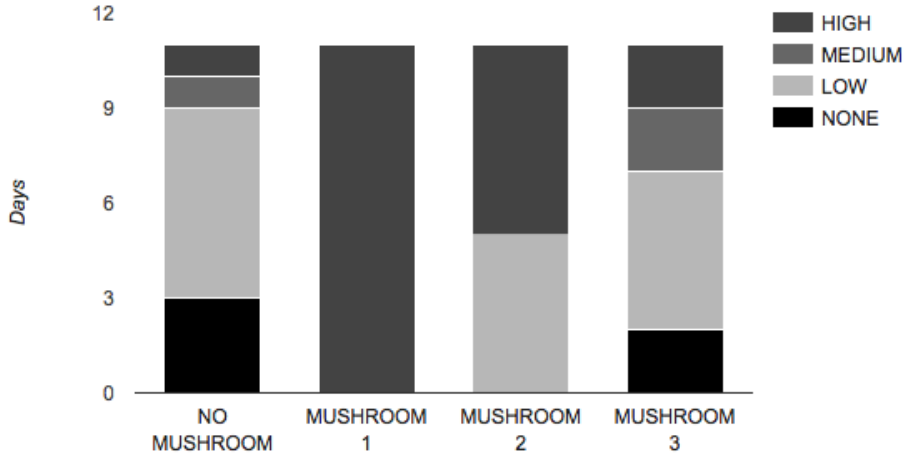

Figure 6. Number of days that each study site showed None, Low, Medium, or High insect species richness. Overall, plots with mushrooms tended to have higher insect species richness than the plot without a mushroom. Control Mushroom is omitted due to lack of insect species richness data. For full Control Mushroom insect species richness data, see Appendices 5 and 8.

Table 1: I ran a Kolmogorov Smirnov test that compared the [H+] concentration distributions for Mushrooms 1-3 against the [H+] concentration distribution of the No Mushroom plot. Each mushroom [H+] distribution is significantly different from the no mushroom distribution. See Figure 2 for these distributions converted to pH.

Mushroom 1	D = 0.818	$p < 0.001$
Mushroom 2	D = 0.909	$p < 0.001$
Mushroom 3	D = 0.81	$P < 0.001$

Table 2: The True ppm value is the known value for pesticide content in my Malathion dilution calculated by mass, and the registered ppm value is the content registered by the test kit. The pH shows the pH of the pesticide dilution before titration. See Figure 3 for organophosphate content registered in soils.

True ppm organophosphate	26
Registered ppm organophosphate	3.5
pH	6.43

DISCUSSION

After applying organophosphate pesticide to four different plots with and without *Trametes spp*. fungi, I found that substrates with mushrooms tended to have significantly higher pH than the substrate without. Even on Day 0, before pesticide application, the mushroom substrates showed substantially higher pH and continued with this trend after Day 1 and Malathion application. Around Day 6, I observed a slight dip and rise in the pH data of all mushrooms. Around this day, the daily heavy rain started to let up and the days got dryer, and since rain can often influence pH (Mickelbart *et al*. n.d.), I believe this pH change can be attributed to rainfall. However, even with this pH decrease and rise, mushroom pH still remained higher than the no mushroom substrate. Higher pH means lower [H+], therefore substrates with the presence of *Trametes spp*. fungi are more basic than substrates without. This high pH within could indicate that the fungi are aiding in bioremediation. Hydrolysis is an important degradation reaction and is one of the main pathways for organophosphate degradation in soils; it serves to make organophosphates inert by splitting the pesticide molecules apart into phosphate esters and alcohols by reaction with water (Freed *et al*. 1979). This reaction is often most successful in soils with a high pH (Munnecke 1979), as the enzymes that contribute to hydrolysis are catalyzed (Singh and Walker 2006). Therefore, hydrolysis of organophosphates is more likely in these substrates than the substrates without *Trametes spp*. From this, I would like to further test to see if *Trametes spp*. could be altering their substrate pH rather than just showing preference for high pH substrates for growth. If these mushrooms are in fact able to actively change their substrates' pH, it is more likely that they could be useful bioremediators.

The pH is also an interesting factor in mushrooms, as many species have been observed to be more successful in soils of lower pH (Rousk *et al*. 2009). Thus, when using *Trametes spp*. mushrooms to bioremediate organophosphate polluted soils, it may be important to find a balance between pH high enough to destroy the pesticides and pH low enough to allow for other

mushroom growth. The data collected on organophosphate content in the substrate varied substantially and did not behave as expected. On Day 0, before applying pesticides, pesticides were registered with the kit, and on Day 1, only mushrooms 2 and 3 behaved as expected with an increase in substrate pesticide content. After Day 1, all data is very scattered and a trend is difficult to discern. Because the data was so scattered, I performed a test of accuracy on the organophosphate test kit. The test kit registered only 3.5 parts per million organophosphate content in my solution when the actual value was 26 parts per million. This large discrepancy in pesticide content led me to attribute organophosphate data inconsistencies to potential test kit error. This error could have been due to iron and sulfate presence in the substrates as well, as the test kit lists these as two compounds as having the potential to interfere with titration data. For future tests, I would like to find a more accurate way of testing organophosphate content in order to measure rates of pesticide degradation. With an accurate rate of degradation, I could test Stamets' hypothesis that *Trametes versicolor* actually contributes to organophosphate breakdown (Stamets 2005).

Insect presence and species richness also served as an indicator of potential pesticide presence. Since Malathion is marketed as an insecticide for all insects, I hypothesized that it would kill insects on the substrates to which it was applied, and thus greater insect presence should correlate to less pesticide. Surprisingly though, there was no real decrease in insect presence on the substrate with the mushrooms— insect presence and species richness remained relatively high throughout the length of the study compared to the no mushroom substrate. Based on this data, I propose that the insecticide was less effective in killing insects on the substrates with mushrooms than the substrate without. This could be due to the mushrooms creating a more insect-friendly habitat that is easier to recolonize after pesticide application, or it could potentially be due to other factors, such as quality of substrate or presence of territorial insects. Further studies should be conducted to compare effects of pesticide on insects on *Trametes spp.* substrates versus no *Trametes spp.* substrates.

In conclusion, organophosphate breakdown by hydrolysis is more likely on the substrates of *Trametes spp.* fungi than on substrates without because of the higher pH of mushroom substrates. Malathion also seems to be less active on these mushroom substrates based on insect presence and species richness data. Both of these observations could indicate that *Trametes spp.* fungi have potential as bioremediators if further testing confirms a cause and effect relationship between the mushrooms and the altering of organophosphate contents. Rates of organophosphate breakdown may be helpful in discerning this, as well testing for compounds excreted by the fungi that can alter pH. Based on this data, I hypothesize that *Trametes spp.* fungi do in fact aid in the bioremediation of soils contaminated by organophosphate pesticides by altering pH, and further testing could confirm this. *Trametes spp.* fungi could very well be a viable option for underprivileged communities that need a cheap, natural, and safe option to rid their soils of unsafe residues of organophosphate pesticides from drift.

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