Soil Ecology Project:

The Effects of Acid Rain on Fungi in the Soil

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Background Report

Acid rain is a type of precipitation that is the result of air pollution caused by chemicals produced by the burning of fossil fuels, such as coal, oil, and natural gas (Pelley, 2015) "Acid Rain," n.d.). The gases emitted from these processes, nitrogen oxides and sulphur dioxide, react with the droplets of water in clouds to form nitric and sulphuric acid that then falls as “acid rain,” and the strength of its acidity depends on the degree of air pollution and air quality in a specific location. While all rain is slightly acidic due to the chemical reaction between water and the CO_2 in the air (with a pH of about 5.6) (Likens, 2007), air polluted with nitrogen oxides, sulphur dioxides, and hydrocarbons can increase the acidity of precipitation to a pH of as low as two ("Acid Rain," n.d.).

Although acidic conditions are common in nature, the additional hydrogen ions present in acid rain and other forms of acidic precipitation are harmful because of the way these ions can affect the environment (Schenabel, 2017). When the acidic rain soaks into the soil, the extra H^+ decreases the amount of nutrients available to the plants by dissolving key minerals such as nitrogen, potassium, and phosphorus, causing them to wash away before said plants can use them to grow (McKenzie, 2003). In addition, the extra acid in the soil can also cause the release of toxic substances into the soil, such as aluminum, that are very harmful to trees and plants ("Effects of Acid," n.d.).

Given that acid rain impacts the mineral content of the soil so significantly, it is also therefore likely to have an impact on the many microorganisms that live there. One such group of microbes are the fungi. These heterotrophic organisms release chemicals that can digest biological matter which the fungi then absorb for their own individual energy needs. Fungi extend thread-like extension of their cells called hyphae which release enzymes in order to for

the fungi to extract nourishment from these dead organisms.(Featherstone, n.d.). Thus, one of the main functions of fungi in the soil ecosystem is to decompose and recycle materials and to distribute nutrients for other organisms living in the soil to absorb for their own metabolic needs ("Characteristics of Fungi," 2016).

One of the most common and most important of these decomposing fungi are the mycorrhizae. These fungi are crucial to plants (Pace, n.d.), and while there are a variety of species, the two main categories are the Arbuscular (or endomycorrhizae) and the ectomycorrhizae. Arbuscular mycorrhizae actually physically penetrate the cells of their host's roots, whereas ectomycorrhizae surround the roots in a net-like structure that embraces the roots without directly touching them. Most plants will host one of these two categories of fungi ("Mycorrhizae," n.d.), and together this relationship allows the plant to absorb water and nutrients more efficiently, while in return the fungi receives carbohydrates from the plant's photosynthetic processes. Specifically, fungi are able to absorb the phosphorus and nitrogen from the soil around them, which plants often have difficulty doing (Pace, n.d.), thereby preventing these key nutrients from escaping to where the plants no longer have access to them ("Mycorrhizae," n.d.). Furthermore, mycorrhizae increase protection for their host plant, enabling them to fight off certain soil pathogens. Therefore, mycorrhizae are critical to the survival of plants because they enable plants to take in water, phosphorus, and nitrogen more easily ("Mycorrhizas," n.d.) (Pace, n.d.).

Yet how might acid rain impact these crucial soil microbes? The answer lies in the relationship between how well an enzyme functions and the pH of the environment. The three dimensional structures of proteins are very sensitive to any changes in the surrounding pH, and the shape of enzymes will change based on how acidic or basic the environment is. When the pH

level of the environment gets too acidic, the shape of the enzyme denatures, altering its ability to complete its functions. But since enzymes are responsible for starting and stopping chemical reactions between the biological molecules that means that when enzymes are unable to complete this function, the necessary cell processes does not take place, and since a fungus, just like any other organism, is made of cells, this means the fungus would die (Reece et al., 2014, pp. 155-156). Thus the potential danger of acid rain for fungi and, as a consequence, for the larger ecosystem is that the fungi could possibly die meaning that everything in the ecosystem would be negatively impacted and be likely to die as well.

Therefore, we wanted to see what the effects of acid rain on the fungi in the soils at our school might be. Acid rain is known to cause damage to the soil, thus we were curious as to whether it would affect the mold and yeast count in the soil. We hypothesised that the acid rain will cause fungi in the soil to die. We set out to test this prediction by applying sulfuric acid to the soil plots on our campus and determining if the density of fungi in the soil would change.

Lab

I. Question: Does acid rain change the density of fungi in the soil?

II. Hypothesis: When acid rain interacts with the soil, the density of fungi living in the soil will decrease.

III. Procedure:

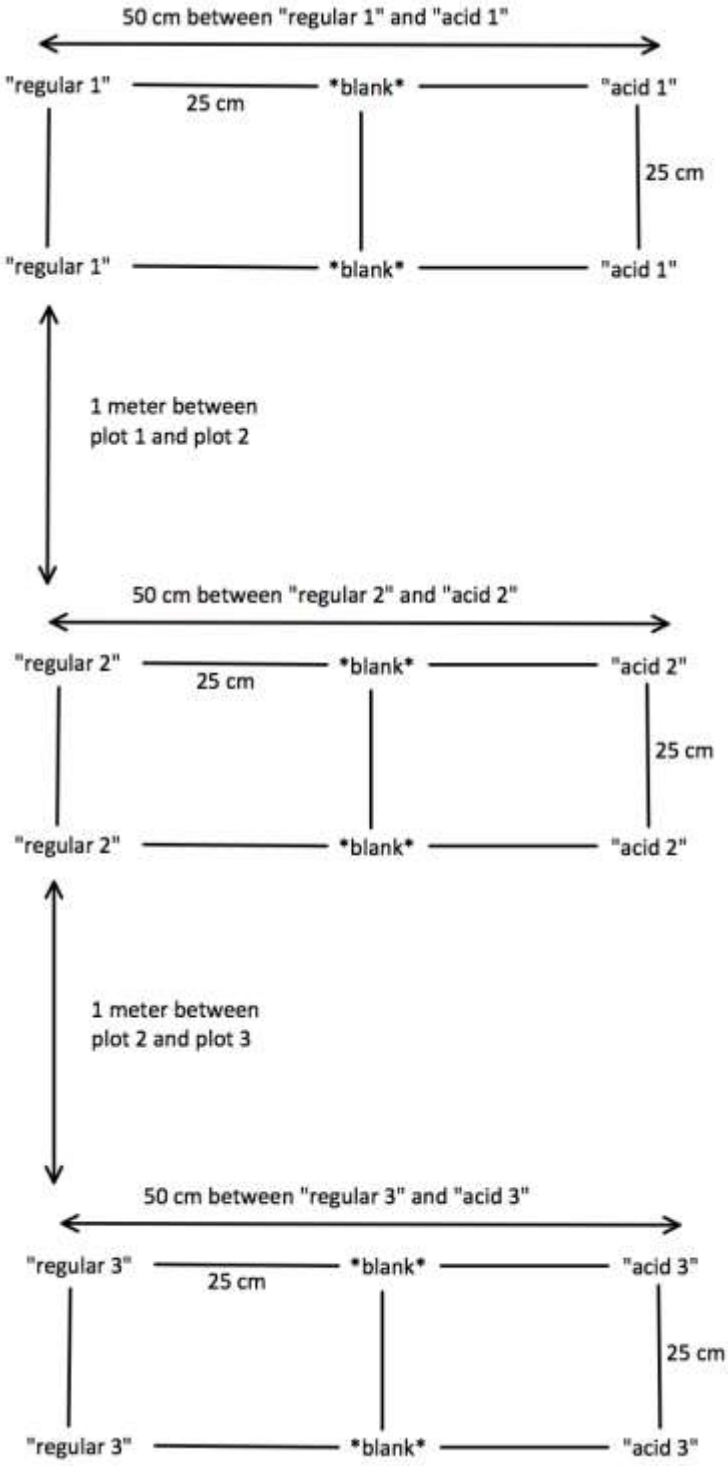
- Independent variable: the addition of acid rain to a soil plot
- Dependent variable: the density of fungi in the soil
- Negative control: the addition of distilled water to a soil plot
- Positive control: the soil samples before adding the acid solution and the distilled water
- List of controlled variables:
    - General
        - The longitude and latitude of the plots
        - The size of the plot/ spacing between flags
        - The distance each plot is from each other
        - The pH level of the water-sulphuric acid solution
        - The amount of water and sulphuric acid solutions being poured on each soil plot
        - How long the acid rain is on the soil for before being extracted and tested (about 48 hours)
        - How much soil is being extracted from each plot from the ground
        - The depth and diameter of the soil being extracted

        - Make sure all samples are collected on the same day at the same time
    - Fungi test
        - Size of pipette used to add sterile water to culture tubes
        - Type of pipette used to add sterile water to culture tubes
        - Type of tube containing soil-water solution (culture tube)
        - Power of 10 the soil-water solution is diluted to
        - Type of water and how much used
        - Make sure each set of soil samples (before and after) go through the serial dilution process on the same day at the same time
        - Size/type of agar plates
        - Where agar plates are put to rest
        - Amount of dilution plated
        - Which dilutions are plated
        - Amount of time agar plates grow (72 hours)
        - Size/type of cc scoop
        - Size/type of culture tubes

- Step by step instructions:

1. Get 16 yellow flags, label two of them “regular 1”, two of them “regular 2”, two of them “regular 3”, two of them “acid rain 1”, two of them “acid rain 2”, two of them “acid rain 3”, and leave four of them blank
2. Go to the area of soil on the Southern side of Roland Park Country School at N 39°21.412’, W 076°38.175’

3. Use the labeled flags to make test plots at this location according to the following diagram:



4. Complete the following steps 5-11 on the same day at the same time.
5. Go plot 1 and use the soil core extractor and mallet to dig up 15 cm deep and 2 cm wide of soil. This soil should come from within the square of ground made up by the “regular 1” and blank flags
6. Put the soil into a plastic bag and label “regular 1 soil before”
7. Repeat steps 5-6 2 more times (3 extractions total)
8. Repeat steps 5-7 at plot 2 and plot 3, labeling each bag appropriately based on which plot the soil in that bag came from
9. Repeat steps 5-8 in the squares made up by the “acid _” flags and blank flags based on its respective plot. Make sure each of these bags is labeled appropriately: “acid _ before”
10. After following steps 5-9 there should be 18 bags of soil total, 3 “regular _ soil before” and 3 “acid _ soil before” from each plot.
11. Bring all the bags containing soil back to the lab station, set them aside.
12. Immediately before following step 13, mix all 3 bags from each location into a single bag. For example: all 3 bags labeled “regular 1 before” will be combined into a singular bag. Repeat this with the sets of bags from each location. You should now have 6 total bags of soil.
13. On the same day at the same time, complete the following steps 14-24. Do this with the soil from the bags from step 12. Each bag of soil is done separately.
14. With the “reg 1 before” bag of soil, use a clean, new 10 mL serological pipette to add 10 ml of sterile water to a 15 ml culture tube. Label the tube “Reg 1 before 10°”.

15. Use the same pipette to add 9 ml of sterile water to a second 15 ml culture tube. Label the tube "Reg before 10^{-1}"
16. Repeat step 15 one more time to an additional 15 ml culture tube, and label it "Reg before 10^{-2}"
17. Place 1 cc of the soil sample from "the reg 1 before" soil into the "10°" culture tube.
18. Cap the tube and shake vigorously.
19. Using a new serological 10ml pipette, remove 1ml of the soil-water mixture from the "10°" tube and place into the "10^{-1}" tube
20. Cap and shake vigorously
21. Using the same pipette from step 19, remove 1 ml of the soil-water mixture from the "10^{-1}" tube and place into the "10^{-2}" tube.
22. Cap and shake vigorously.
23. Place 100 µl samples from the 10^{-0}, 10^{-1}, and 10^{-2} onto their own separate, correspondingly labeled 3M Petrifilm™ Yeast and Mold Count Plates. The plates should be labeled "Reg 1 before 10^{x}" (x being the dilution value of the soil-water mixture being added to each nutrient agar plate)
24. Repeat steps 14-23 with the rest of the remaining 5 soil bags, labeling each of their test tubes and nutrient agar plates correspondingly to the bag the soil came from and their proper dilution value.
25. Allow the soil-water mixtures on the petrifilm yeast and mold count plates to grow for 72 hours. There should be 18 total petrifilm yeast and mold count plates with soil-water mixture on them.

26. After 72 hours of growth, and on the same day at the same time, examine all the nutrient agar plates. Start by observing (with a magnifying glasses) the plate with the lowest dilution value and count the number of yeast colonies. If the plate with the lowest dilution has no yeast colonies, move up to the plate with the next lowest dilution value until yeast colonies are found. Record the plot information, the number of colonies, and the dilution value of the plate those colonies were found on.
27. Now, repeat step 26 by observing the plates for mold rather than yeast.
28. Record the estimates of the number of fungi colonies in the original 1 cc soil sample using the following formula: # Microbes in 1 cc of soil = # Colonies on sheet x 10^2 x $10^{|\text{dilution \# which these colonies were found}|}$. Record data in data table. Properly dispose of these nutrient agar plates after data has been recorded.
29. Fill a Nalgene bottle with 1000 mL of sulphuric acid that has a pH of 2
30. Label this Nalgene bottle which now contains the lab-made acid rain and set aside
31. In a separate nalgene bottle, pour in 1000 mL of distilled water, label, and set aside
32. Follow the following steps 33-34 on the same day at the same time
33. Go to plot 1 with the two Nalgene bottles containing the acid rain and the distilled water, as well as bringing a graduated cylinder. Using the graduated cylinder, pour 300 mL of acid rain on the ground within the square made up by the “acid 1” flags and the blank flags. Immediately after pouring the acid rain, use a separate

graduated cylinder to pour 300 mL of distilled water on the ground within the square made up by the “regular 1” flags and the blank flags. All pouring should be done by drizzling the liquid from the graduated cylinder over the entire square of soil until the graduated cylinder is empty. The liquid should be, for the most part, evenly dispersed over the square of land.

34. Repeat step 33 at plot 2 and plot 3, making sure the acid rain is only poured in the 25x25 cm squares made up by the “acid _” flags and blank flags and the distilled water is poured within the square made up by the “regular _” flags and blank flags. Also make sure that the one graduated cylinder is designated for pouring the acid rain and the other one is designated for pouring the distilled water.
35. Let the acid rain and the distilled water remain on the soil in plot 1, 2 , and 3 for 48 hours
36. Complete steps 37-43 on the same day at the same time after the acid rain and distilled water has been on the soil for 48 hours
37. Go to the “regular” square of plot 1 and use the soil core extractor and mallet to dig up 15 cm deep and 2 cm wide of soil. Repeat this 2 more times (3 extractions total). This soil should come from within the square of ground made up by the “regular 1” and blank flags
38. Put each soil extraction into their own plastic bag and label “regular 1 soil after”
39. Repeat steps 36 and 37 with the “regular” squares at plot 2 and plot 3, labeling each bag appropriately based on which plot the soil in that bag came from. These are all soil extractions from the 25x25 cm squares made up of “regular _” and blank flags. Make sure each bag is labeled appropriately: “regular _ soil after”.

40. Repeat steps 37-39, but with the soil extractions coming from the 25x25 cm the squares made up by the “acid _” flags and blank flags based on its respectable plot. Make sure each bag is labeled appropriately: “acid _ soil after”.
41. After following steps 37-40, there should be 18 bags of soil total, 3 “regular _ soil before” and 3 “acid _ soil before” from each plot.
42. Bring all the bags containing soil back to the lab station, set them aside
43. Immediately before following step 44, mix all 3 bags from each location into a single bag. For example: all 3 bags labeled “regular 1 after” will be combined into a singular bag. Repeat this with each set of bags from each location
44. Now, on the same day at the same time, follow steps 13-26, but with the soil bags from 37-43. Make sure each bag of soil is done separately. Make sure each test tube and nutrient agar plate are properly labeled with “after” rather than “before” and the correct dilution value and plot #.

Citation; LaMotte STH Series 14

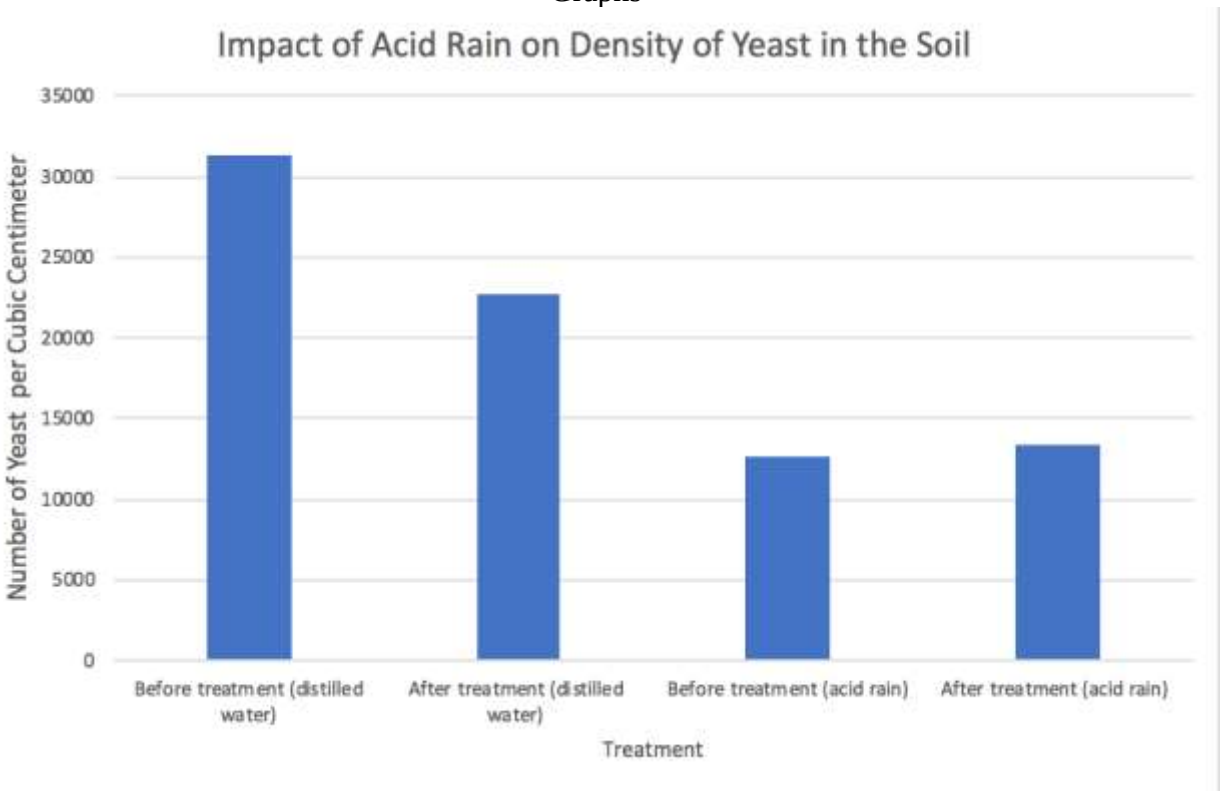
IV. Data Table and Graphs

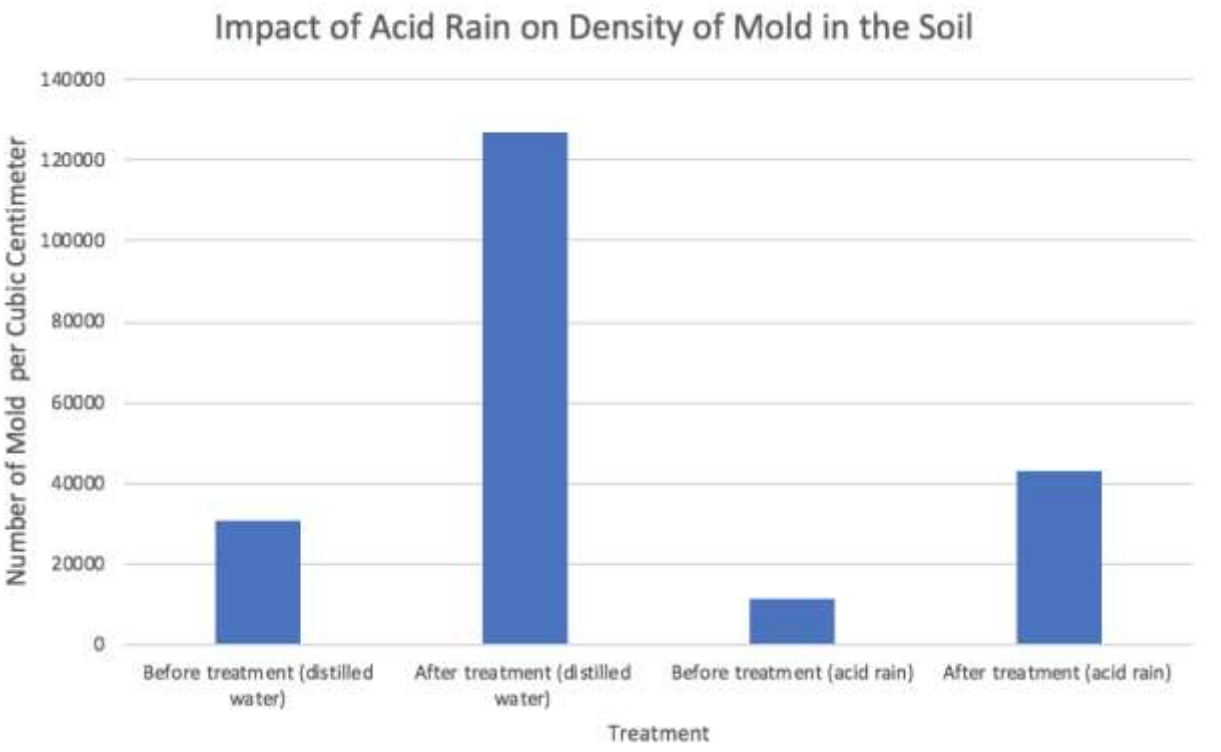
Data Table

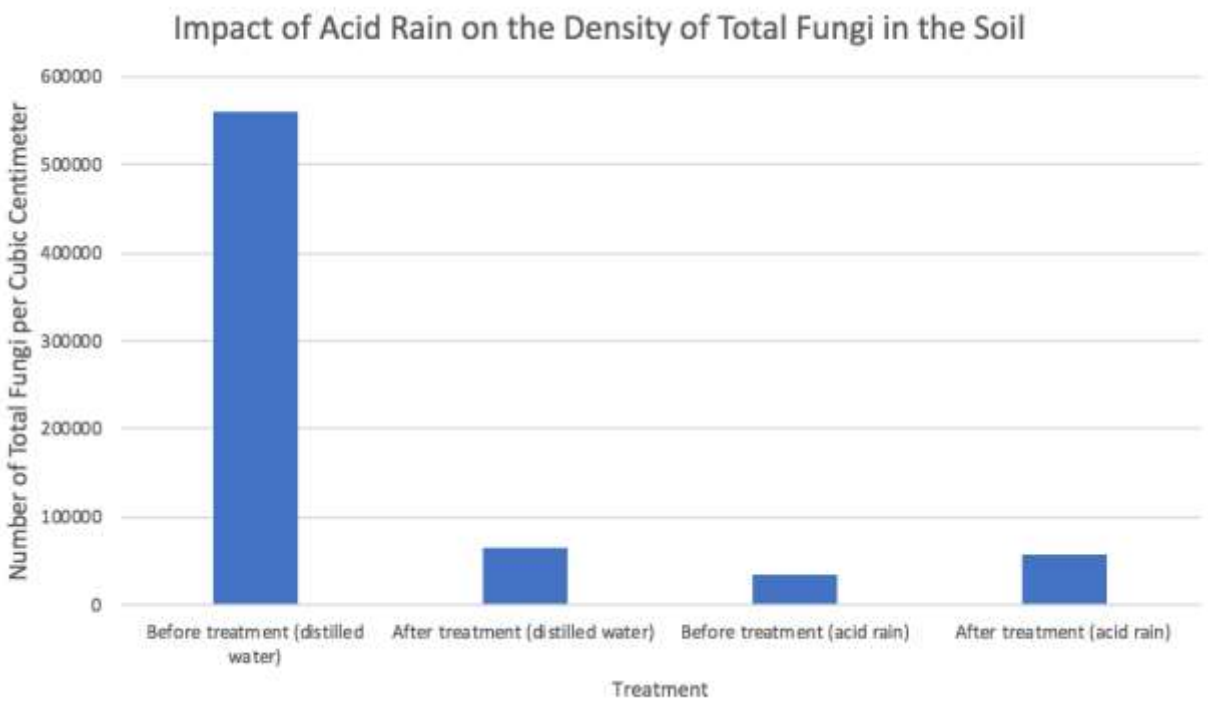
Impact of Acid Rain on the Density of Soil Fung (number per cubic centimeter)

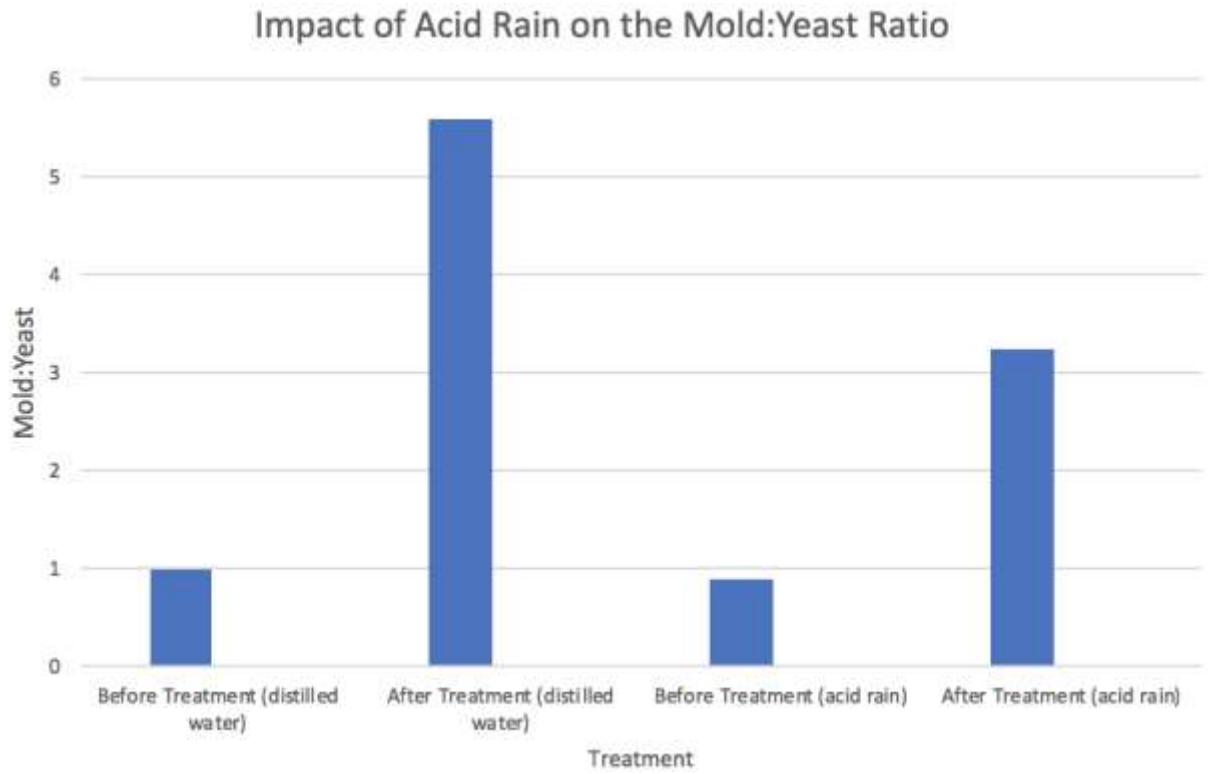
Trial number	Application of Distilled water on Soil Plots						Application of Acid Rain on Soil Plots					
	Yeast		Mold		Total value		Yeast		Mold		Total value	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
1	30,000	20,000	10,000	50,000	40,000	70,000	8,000	10,000	4,000	30,000	12,000	40,000
2	60,000	40,000	80,000	40,000	140,000	80,000	20,000	10,000	20,000	10,000	40,000	20,000
3	4,000	8,000	3,000	37,000	7,000	45,000	10,000	20,000	10,000	90,000	50,000	110,000
Average	31,333.3	22,666.6	31,000	127,000	561,000	65,000	12,666.6	13,333.3	11,333.3	43,333.3	3,4000	56,666.6

Graphs


Impact of Acid Rain on Density of Yeast in the Soil
Number of Yeast per Cubic Centimeter
35000
30000
25000
20000
15000
10000
5000
0
Before treatment (distilled water)
After treatment (distilled water)
Before treatment (acid rain)
After treatment (acid rain)
Treatment


Impact of Acid Rain on Density of Mold in the Soil
Number of Mold per Cubic Centimeter
140000
120000
100000
80000
60000
40000
20000
0
Before treatment (distilled water)
After treatment (distilled water)
Before treatment (acid rain)
After treatment (acid rain)
Treatment


Impact of Acid Rain on the Density of Total Fungi in the Soil
Number of Total Fungi per Cubic Centimeter
600000
500000
400000
300000
200000
100000
0
Before treatment (distilled water)
After treatment (distilled water)
Before treatment (acid rain)
After treatment (acid rain)
Treatment


Impact of Acid Rain on the Mold:Yeast Ratio
Mold:Yeast
6
5
4
3
2
1
0
Before Treatment (distilled water)
After Treatment (distilled water)
Before Treatment (acid rain)
After Treatment (acid rain)
Treatment

Conclusion

According to our data, our hypothesis was incorrect. We predicted that when acid rain interacts with the soil, the density of fungi living in the soil will decrease. Our results prove the opposite, and show that the density of the total fungi living in the soil increased when the acid rain was added to it. When we poured this sulphuric acid rain solution on the soil, the total fungi count went up by 22,666.6, and in fact the fungi count went down in the plots where the acid rain wasn't poured. Although this disproves our hypothesis, our results still show that the acid rain had a negative impact on the soil fungi.

In our negative control plots where, we poured distilled water on the soil, our data proves that it was, metabolically, easier to live as a mold. Everything that allows fungi to be in their most reproductive and healthy forms was present, which is why the mold count went up drastically by 96,000 through the course of our experiment. It is also why the yeast count, or the weaker and less protected fungi form, decreased by 8,666.7. The mold:yeast ratio also had a large increase of 4.61. When fungi have all the nutrients and necessities they need to live readily available, they become mold as opposed to yeast, which is shown in these results and allows us to see that the lack of acid rain had a positive impact on the health, quality, and condition of the fungi. Most likely, these substantial results were due to the immense amounts of rain that fell during our experiment. Fungi prefer living in moist environments which is why the rainfall enabled them to thrive. But, despite the positive impact of the rain on the health of the fungi, the total count of fungi in the negative control plot decreased by 496,000. This remarkable change in the data's pattern allow us to conclude that there was something else attacking the fungi, which was the reason behind their deaths. In the negative control plot, it became easier for a fungus to survive in its healthiest form due to environmental conditions, but an outside invader was

competently eating the fungi, causing them to die and making the total fungi count decrease immensely.

The data results of the acid rain plots confirm that the acid rain had an impact on the health of the fungi in the soil. This is shown through the data because there weren't as many fungi in their healthiest form as there were in the negative control plot. Although, the graphs for the impact that the acid rain had on the density of yeast, fungi, and the mold:yeast ratio suggest that even with the acid, the fungi were able to be in a slightly better metabolic form. More specifically, this density of yeast increases by 666.7, the density of mold went up by 32,000, and the ratio of mold:yeast went up by 2.355. Because the mold count and the total fungi count increased by so much, this data shows that the fungi were healthy and thriving in their environment. Because the data for mold density and the mold:yeast ratio didn't go up as much as they did in the negative control plots, just as the data for the yeast density increased in the acid plots as opposed to the decrease in the negative control plots, the acid is verified that the acid rain still had an impact. But, despite the negative impact that the acid had on the fungi's health, throughout the experiment the total fungi density in the acid rain plot increased by 22,666.6. This information suggests that something about the acid was protecting the fungi from getting eaten and killed. While the acid did make the life and health of the fungi rougher, it withheld the fungi from dying.

After analyzing the data we received from both our negative control and acid rain plots, we were able to assume that an outside invader, one that most likely prefers wet environments such as the much rained on soil of our experiment plots, was eating the fungi. But we also know that the presence of the acid rain in the soil protects the fungi from this intruder, meaning that the invader is susceptible to harm and/or death resulting from sulphuric acid rain. We determined

that the most logical attacker is protozoa were eating the fungi. Protozoa are therefore the explanation behind the catastrophic decrease in the total fungi density within the negative control plots, while also being inhibited by the acid rain making them no longer able to eat the fungi. Protozoa are single celled organisms that live in the thin film of water that lines the pores of the soil. Protozoa are fungivores, meaning they feed on fungi. Protozoa need water in order to function. When soil dries out, the water films where the protozoa live evaporates. When that happens and the water is gone, the protozoa that live in those films are forced to change shape and become cysts. That means they are trapped in an immobile, inactive state. They have to remain in that state until the water comes back. Essentially, protozoa are more prevalent and active in the soil when there is more water (Nardi, 2003, pp. 53-54). Because it rained so heavily while we were testing our soil plots, the protozoa living in the soil were able to be very active. Using our knowledge about protozoa, we are able to conclude that protozoa were most likely what killed the fungi in our soil plots during our experiment. To prove this theory, we would need to conduct another experiment in which we would test for protozoa in the soil. It would be the same as our last experiment, testing for the density of fungi in the soil, but with a few changes. We would still be testing for the fungi density in the soil, but we would also be testing for the amount of protozoa in the soil. We would also control for the amount of additional rainfall that the soil receives. In our negative control, the soil would receive no additional water besides the water we pour onto it. But we would also test the soil with an overwhelming extra flow of rain, since that is what we think caused the protozoa to be so active in the soil during our experiment. We hypothesize that when there is a lot of water falling onto the soil, the overall fungi count will go down because once again, the protozoa will eat the fungi.

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