The Effect of Nitrogen-Based Fertilizer on Bacteria Diversity in the Soil

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Background:

Bacteria are among the smallest yet most plentiful microbes in soil, and there can be as many as 100 million to 1 billion bacteria, with as many as 60,000 different species, in a single teaspoon of soil. Among these many types, some of the major categories include decomposers, nitrogen-cyclers, disease-suppressors, sulfur oxidizers, and actinobacteria; each of them playing their own critical role in the soil ecosystem. The first of these, the decomposing bacteria, break down organic materials within the soil during the earlier stages of decomposition (before the soil fungi take over); while the disease-suppressing bacteria provide plants protection against numerous soil-borne pathogens. The sulfur oxidizing bacteria transform sulfides into sulfates, a form of sulfur that plants can access and use to promote protein production, and the actinobacteria promote nutrient uptake by plants by slowly breaking down humic acids and humates within the soil (Reid, 2005).

However, the most significant group of bacteria that live in the soil are those involved in the nitrogen cycle. This biogeochemical process "circulates nitrogen in various forms through nature" (Encyclopedia Britannica, 2018) and consists of three main steps: nitrogen fixation, nitrification, and denitrification. In the first step, bacteria in the soil collect nitrogen gas from the air and convert it into a form of nitrogen called ammonium which plants can absorb through their roots. They do this because the largest source of the element nitrogen occurs as nitrogen gas in the atmosphere (78%) and is inaccessible to most organisms on this planet, including most bacteria. But the nitrogen-fixing bacteria can work with nitrogen gas to benefit themselves, the plants, and the other living things in the soil around them. In fact, some nitrogen-fixing bacteria form direct symbiotic relationships with the roots of plants, where the plants

supply the bacteria with the sugar they need to make the ATP energy that allows the nitrogen-fixing bacteria to produce the ammonium for the plants (Deacon, 2018).

Ammonium, though, is a toxin for most living things, and so the next step of the nitrogen cycle is nitrification, where nitrifying bacteria convert the excess ammonium in the soil to nitrite and then nitrate, which can also be easily absorbed from the soil by the plants. Finally, denitrifying bacteria metabolize any remaining nitrate back into nitrogen gas or nitrous oxide and return it to the atmosphere (Encyclopedia Britannica, 2018). Hence, the bacteria involved in the nitrogen cycle are like traders in a stock exchange, "buying" and "selling" the different forms of nitrogen that the different organisms in the soil require at any given moment in the life of the ecosystem. Nitrogen acts as an ecological currency, and just like in the world of banking, stop cycling the nitrogen, and the entire ecosystem crashes.

The reason that it crashes is because nitrogen is a vital element to both growth and reproduction in plants and animals. It is critical to the formation of nucleotides, which make up DNA and RNA, and amino acids, which make up proteins. Without DNA, RNA, and proteins, there are no enzymes to start and stop the chemical reactions that allow cells of any kind to function. Therefore, without nitrogen, cells cannot survive, and the organisms made out of them will die, meaning the plants in any given location die and any life based on consumption of those plants will die. In other words, without nitrogen, an entire ecosystem collapses and fails.

Because nitrogen is so essential for organisms to survive, people have developed additional methods to help plants obtain the nitrogen they need in order to improve crop production. One such way is through nitrogen-based fertilizers. Fertilizer, by definition, is "a natural or artificial substance containing the chemical elements that improve growth and productiveness of plants. Fertilizers enhance the natural fertility of the soil or replace the

chemical elements taken from the soil by previous crops" (Encyclopedia Britannica, 2017). People use artificial fertilizer when plants have not been growing quickly enough to meet their needs, and indeed, the sole purpose of fertilizer is to make plants grow larger and faster. That is why the three main ingredients that go into fertilizer are nitrogen, potassium, and phosphorus. Nitrogen's role is the most important because it provides for proteins for the plant (e.g. the critical molecule chlorophyll, which allows the plant to photosynthesize and produce its own energy). Potassium is necessary meanwhile in high amounts because it is a nutrient essential for plant growth, and phosphorus is the nutrient that allows plants to convert nutrients into the actual molecular building blocks with which to grow (Encyclopedia Britannica, 2017; BBC, 2014).

What is significant about the nitrogen in the fertilizer is that it is converted by the soil bacteria into usable forms much the same way it is in the natural nitrogen cycle. However, when bacteria within soil convert nitrogen gas to ammonium, the bacteria get rid of the ammonium as quickly as possible because ammonium is their waste and is therefore toxic to them. Hence, by adding excess nitrogen through nitrogen-based fertilizer, the bacteria have to convert it rapidly to ammonium since that is their function in the environment. However, doing so causes them to begin inhabiting significantly more of their own waste (since the nitrifying bacteria as well are overwhelmed by all the excess ammonium now in the soil), and the nitrogen-fixing bacteria can begin to die as the ammonium builds up. As a result, once the plants have grown and been harvested, the remaining bacteria in the soil will not be able to keep up with the normal demands for cycling nitrogen, leaving the soil depleted of this critical element. As a result, the addition of even more nitrogen-based fertilizer is needed the next time for the crop grow (Brock, 2018).

This can become a vicious cycle, and that is why we wanted to explore whether or not nitrogen-based fertilizer affects the diversity of bacteria in the soil. The three types of bacteria involved in the nitrogen cycle - nitrogen-fixing, nitrifying, and denitrifying - work solely to fix or convert nitrogen; so, if extra nitrogen is present in the soil, our hypothesis is that the bacteria will not work as efficiently as normal and therefore certain species of it will die. Our hypothesis is that this will result in an increase in the bacteria's diversity.

Experiment Outline:

I. Question:
   a. Does the application of nitrogen-based fertilizer increase or decrease the diversity of bacteria in soil?

II. Hypothesis:
   a. The application of nitrogen-based fertilizer decreases the diversity of bacteria in the soil.

III. Procedure:
   a. Independent Variable: application of nitrogen-based fertilizer to soil plots
   b. Dependent Variable 1: density of different species of bacteria in soil plots
   c. Dependent Variable 2: nitrate levels in ppm
   d. Negative Control: soil plots with only water added
   e. Positive Control: soil samples collected before fertilizer/water application
   f. Controlled Variables:
      1. Location of soil plots- N 39° 21.483', W 076° 38.178'
      2. Size of soil plots- 30cm X 30cm

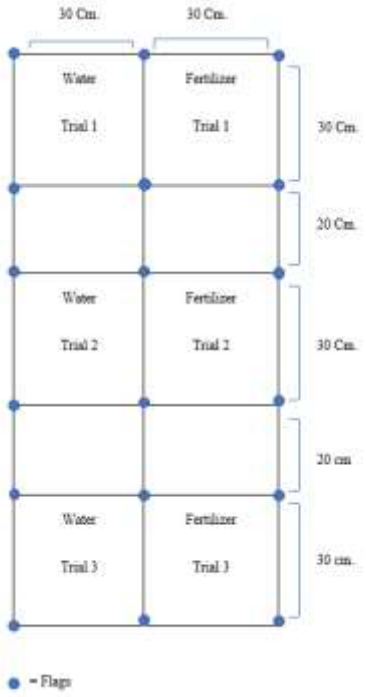
3. Distance between different trial plots- 20cm
4. Soil sample size- 2cm diameter, 15cm depth
5. Time soil samples are taken (same time and day to control for weather during the different trials)
6. Concentration of fertilizer added to soil- 1.4 grams per 30cm x 30 cm plot
7. Type of fertilizer- Vigoro Ultra Turf, 29-0-4
8. Amount of water added to soil- 1 liter per 30cm x 30 cm plot
9. Time between addition of fertilizer/water and taking experiment samples- 4 days
10. Size of serological pipette- 10ml
11. Size of culture tubes- 15ml
12. Type of water in culture tube- sterile water
13. Amount of water in initial culture tube (10^0)- 10ml
14. Amount of water in other culture tubes (10^{-1}, 10^{-2}, 10^{-3})- 9ml
15. Amount of soil added to initial culture tube (10^0)- 1cc
16. Amount of mixture taken from previous tube and added to next tube- 1ml
17. Type of nutrient agar plate- 3M Petrifilm™ Aerobic Count Plate
18. Amount of final solution on agar plate- 100 microliters (.1ml)
19. Type of test kit used- LaMotte Model STH-14 Test kit
20. Test performed- nitrate/nitrogen test
21. Degree to which diluted- 10^{-3}
22. Dilution levels plated- 10^{-2} and 10^{-3}

23. Method for identifying bacteria groups- number of small and large colonies
24. Time bacteria is allowed to grow on agar plate- 72+ hours

g. Step-by-Step Instructions:

1. At the coordinates, N 39° 21.483', W 076° 38.178', mark 2 adjacent 30 cm x 30 cm squares of soil using 6 total flags, and label the left plot "Water- Trial 1," and label the right plot "Fertilizer- Trial 1" (see diagram 1)
2. Repeat step 1, 20 cm from the first plot, and label "Water- Trial 2" and "Fertilizer- Trial 2," respectively (see diagram 1)
3. Repeat step 1, 20 cm from the second plot, and label "Water- Trial 3" and "Fertilizer- Trial 3," respectively. (see diagram 1)

Diagram 1:



4. Complete steps 5-7 on the same day, at the same time
5. With a soil core extractor, collect a 15cm deep, 2 cm diameter sample of soil from the “Water- Trial 1” plot and place the soil gathered in a plastic bag labeled “Positive Control Water Trial 1”
6. Repeat step 5 two more times in a different spot within the same plot and place the soil samples into separate plastic bags, labeled the same as the bag in step 5 (with an additional 2 or 3 at the bottom, respectively, depending on the repetition number)
7. Repeat steps 5-6 for each plot that has been laid out (5 more times) and place the samples in their respectively labeled plastic bags (labeled with the plot name as well as ‘Positive Control’)
8. Complete steps 9-30 on the same day, at the same time
9. Combine the three soil samples from each plot into one plastic bag and combine thoroughly
10. Use the LaMotte Model STH-14 Test kit to test for nitrogen/nitrate levels in the “Positive Control Water Trial 1” plot
11. Record the results in the data table in parts per million (ppm)
12. Repeat steps 10-11 5 more times with the 5 other soil samples
13. Use a clean new 10ml serological pipette to add 10 mL of sterile water to a 15mL culture tube. Label the tube with “10^0” as well as “Positive Control Water Trial 1”

14. Use the same pipette to add 9 mL of sterile water to a second 15 mL culture tube. Label the tube with "10^{-1}" as well as "Positive Control Water Trial 1"
15. Repeat step 14 two more times to two additional 15 mL culture tube, only label them with "10^{-2}" and "10^{-3}" as well as "Positive Control Water Trial 1"
16. Place 1 cc of your soil sample from plot "Positive Control Water Trial 1" into the "10^{0}" culture tube
17. Cap the tube and shake vigorously
18. Using a clean new 10ml serological pipette, remove 1 ml of the soil/water mixture from the "10^{0}" tube and place into the "10^{-1}" tube
19. Cap and shake vigorously
20. Using the same pipette as in step 18, remove 1 mL of the soil/water mixture from the "10^{-1}" tube and place into the "10^{-2}" tube.
21. Cap and shake vigorously
22. Using the same pipette as in step 18, remove 1 mL of the soil/water mixture from the "10^{-2}" tube and pace into the "10^{-3}" tube.
23. Cap and shake vigorously
24. You should now have a total of 4 culture tubes.
25. Plate 100microliter samples from the 3rd and 4th tubes ($10^{-2}$and 10^{-3}) onto their own separate and correspondingly labeled nutrient agar 3M Petrifilm™ Aerobic Count Plate
26. Allow to grow for at least 72 hours

27. Examine each of the plated dilutions for one trial for individual bacteria colonies and choose the plate with the lowest dilution but with at least 5 colonies
28. Count and record the number of small colonies and the number of large colonies observed on the plate
29. Use the following formula to determine density of large and small bacteria groups in soil sample:

Microbes in 1 cc of soil = # Colonies on sheet x 10^2 x $10^{|\text{dilution \# at which these colonies were found}|}$

30. Repeat steps 13-29 five more times with each of the 5 other gathered soil samples, labeling the test tubes with the respective dilution and soil plot ("Positive Control Water/Fertilizer Trial _" 1, 2, or 3)
31. Complete steps 31-32 on the same day, at the same time
32. Place 1.4 g of nitrogen-based fertilizer on each of 30 cm x 30cm plots labeled "Fertilizer Trial _" (1, 2, or 3)
33. Pour 1 liter of tap water on each of the 6 plots
34. Wait 4 days
35. Repeat steps 4-30 after the 4 days have passed (this time, when labeling the plastic bags, do not put "Positive Control" preceding the plot type and trial number)

Data/Observations:

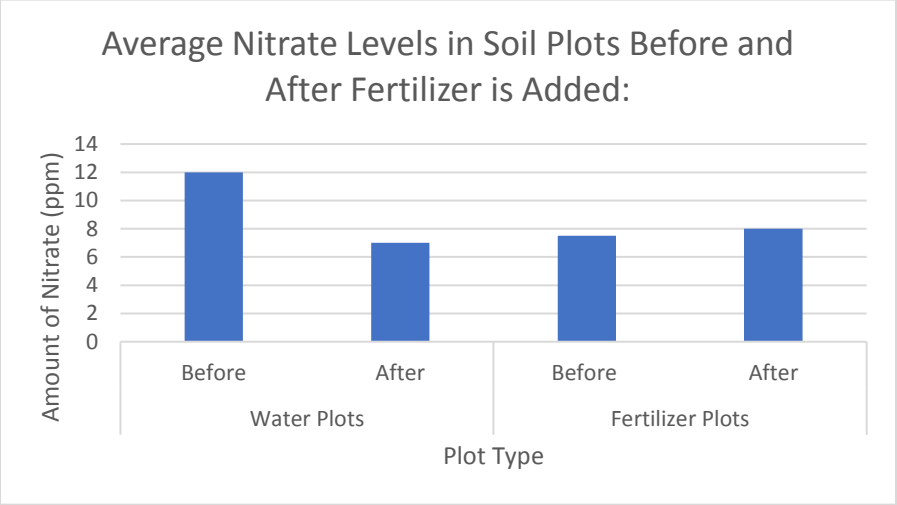
IV. Data & Analysis

Impact of Fertilizer on the Density of Different Species of Bacteria (# Bacteria per 1cc of soil)

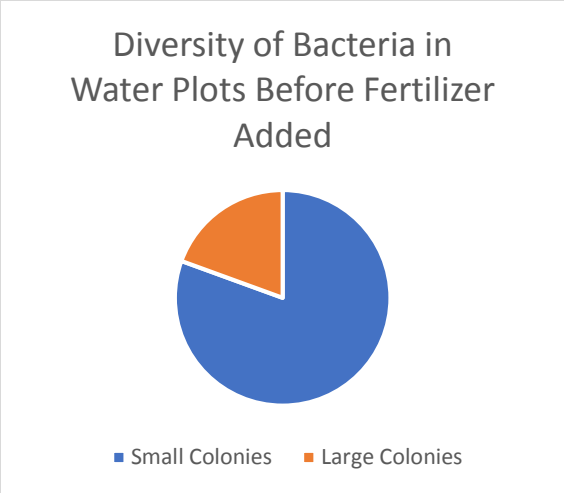
	Before Fertilizer Added					
	Water Plots			Fertilizer Plots		
	Nitrate Level (ppm)	Small Colony Species	Large Colony Species	Nitrate Level (ppm)	Small Colony Species	Large Colony Species
Trial 1:	10	1,000,000	100,000	7.5	800,000	200,000
Trial 2:	10	1,200,000	300,000	7.5	800,000	800,000
Trial 3:	15	700,000	300,000	7.5	700,000	500,000
Average:	12	966,667	233,333	7.5	766,667	500,000

	After Fertilizer Added											
	Water Plots						Fertilizer Plots					
	Nitrate Level (ppm)	Total Colonies	Small Dark Colony Species	Small Light Colony Species	Large Dark Colony Species	Large Light Colony Species	Nitrate Level (ppm)	Total Colonies	Small Dark Colony Species	Small Light Colony Species	Large Dark Colony Species	Large Light Colony Species
Trial 1:	5	86,900,000	86,300,000	0	600,000	0	7.5	54,000,000	53,600,000	0	400,000	0
Trial 2:	5	68,300,000	67,300,000	0	500,000	500,000	10	72,800,000	72,100,000	0	700,00	0
Trial 3:	10	113,600,000	112,700,000	0	0	900,000	7.5	33,600,000	33,200,000	0	400,000	0
Average:	7	89,600,000	88,766,667	0	400,000	500,000	8	53,466,667	52,966,667	0	500,000	0

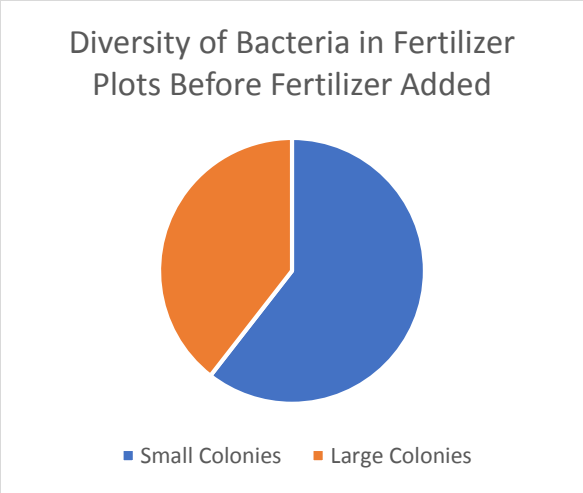
Graph 1:



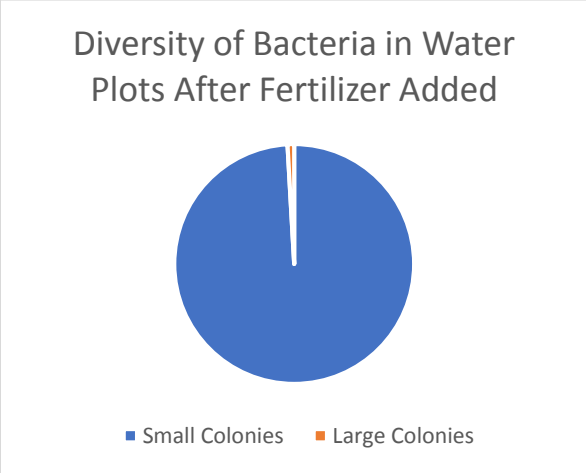
Graph 2:



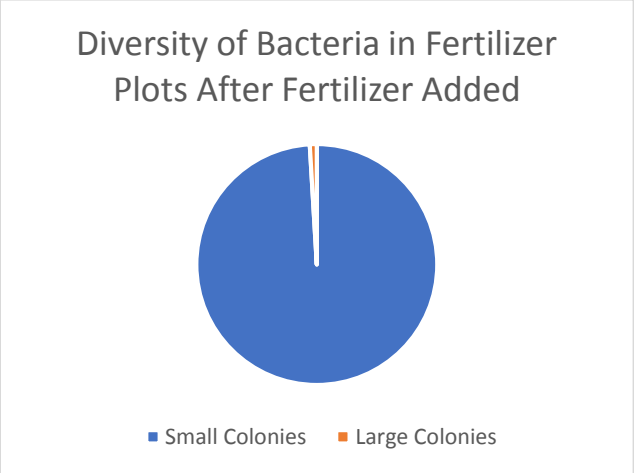
Graph 3:



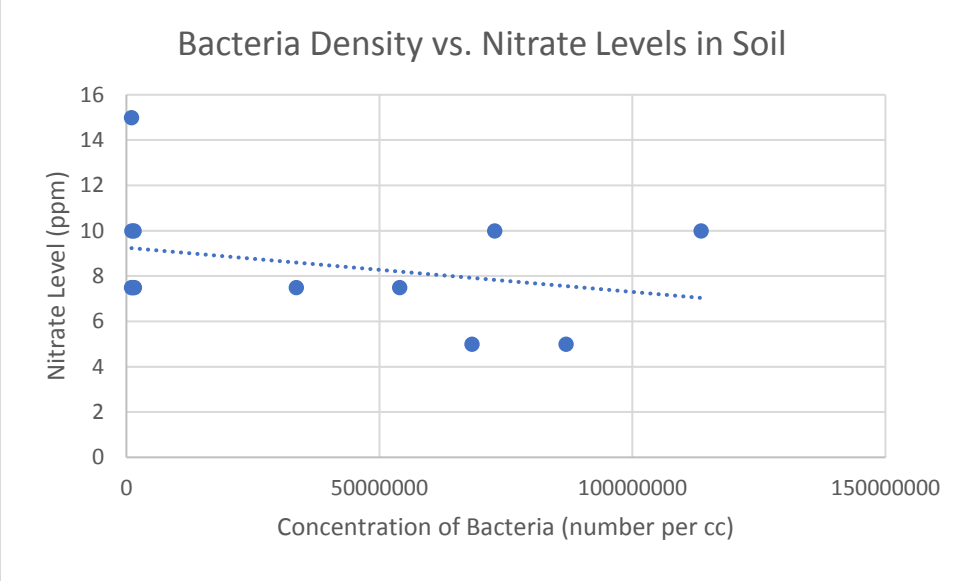
Graph 4:



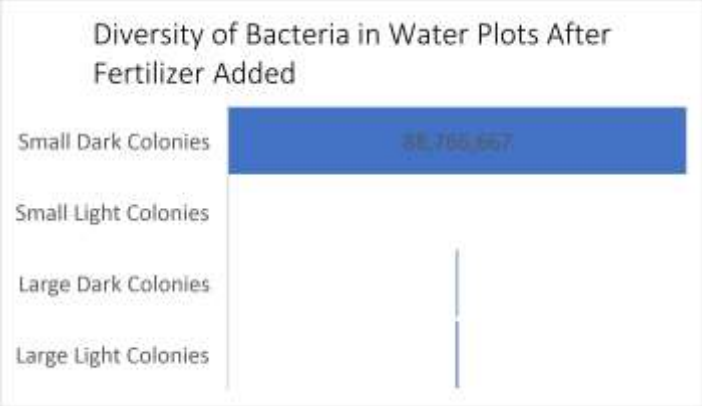
Graph 5:



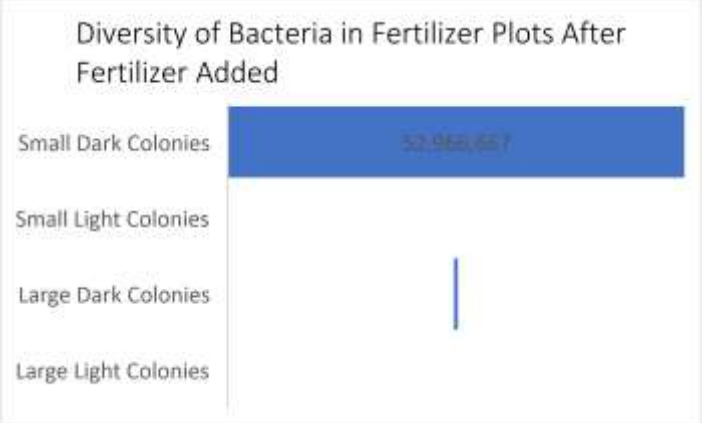
Graph 6:



Graph 7:



Graph 8:



Conclusions:

V. Conclusion

a. Our hypothesis was incorrect- according to our experiment, even though we were successful in manipulating the amount of nitrogen in the soil, we cannot conclude that the application of nitrogen based fertilizer was the cause of the decrease in the diversity of bacteria in the soil. In our graph for nitrate levels, (Graph 1) it shows that we succeeded in increasing the amount of nitrogen in the soil; in the water plots, where the environment remained undisturbed by fertilizer, the amount

of nitrogen in the soil naturally decreased by 5ppm. However, in the fertilizer plots, although according to the graph the nitrate levels appear to have risen by only .5ppm after the fertilizer was added, this increase is much more significant because the natural nitrate levels in the soil dropped considerably during the time our experiment was being conducted, so therefore there was/we caused a substantial increase of nitrogen in the soil. Although these results show that we did increase the amount of nitrogen in the soil, our results shown in graphs 2-6 do not support our hypothesis. A decrease in bacteria diversity is shown between graphs 2 and 4, as well as between graphs 3 and 5- after both trials, an increase in small colonies and a decrease in large colonies (almost all large colonies disappeared) was shown. Since the bacteria diversity decreased in the negative control (water) plots between trials, where we did not add any fertilizer, as well as in the plots where we did add fertilizer, it shows that it was another aspect in the environment that caused this change, rather than the fertilizer. Additionally, in graph 6, although a graph of this type should display that as the amount of nitrate in the soil increases, the number of bacteria in the soil increases, our graph displayed the opposite, which confirms that there is a disruption in the ecosystem where our plots were located. Therefore, since our graphs show that the bacteria diversity decreased in both plots after adding fertilizer to only one of them, and that the level of nitrogen in the soil did, in fact, increase in the plot where fertilizer was added, we can conclude that the nitrogen-based fertilizer did not cause the decrease in bacteria diversity in the soil. For further research about this topic, the next step would be to find out what other environmental factor was

responsible for causing this decrease in diversity, since we have proved that it was not due to the nitrogen-based fertilizer. During our tests, there were large rainfalls that could have been the cause of this decrease; when it rains it creates a more anaerobic environment in the soil, which could be the cause of the decrease in species of large colonies that is shown in graphs 2-5 if the large colonies are an aerobic species of bacteria that requires oxygen to survive. A possibility is that one species that lives in the soil that we tested could be anaerobic, or does not require as much oxygen to survive, which could explain why there was one type of bacteria that was the most dominant. Although we only have the 'after' results due to an error in the data collection of our experiment, graphs 7 and 8 show that after fertilizer was added, there was one species/type of bacteria (that grows in small dark colonies) that was best able to survive in the environment, because it was the species that increased in population and was the most dominant within the entire population, which could mean that that species does not require as much oxygen to survive. One possible way to test this theory would be to take soil samples from the plots and bring them into a lab, where we would then expose a set of them to aerobic conditions while we expose another set to anaerobic conditions. We would complete the same process that we used during this lab for serial dilutions and counting the bacteria colonies on agar plates to find the diversity of bacteria in the soil.

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