The Effects of Insecticides on Bacterial Diversity

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Biology Nine Honors

1 June 2017

Soil Ecology Background

Bacteria are small, microscopic, single celled organisms that live in the soil and are vital to every terrestrial ecosystem. There, they perform three main tasks: improving soil structure, recycling soil nutrients, and recycling water (National Science and Technology Centerm n.d., Hoorman,2016). In the first of these tasks, soil bacteria form microaggregates less than 250 micrometers in size, which consist of mineral particles, plant roots, and humus that are cemented together by the secretions the bacteria release to bind them. (Hoorman, Reeder, Sundermeier, Islam 2017). These microaggregates then create the building blocks that are essential for healthy soil structure, enabling the exchange of gasses and water with the atmosphere. The second task the soil bacteria do, recycling soil nutrients, involves certain types of aerobic bacteria that can decay and decompose dead organisms to release their valuable nutrients back into the ground (Senior 2000), and in the third task, the soil bacteria create gaps and channels throughout the soil that increases water infiltration, thereby increasing the water holding capacity of the soil and helping to prevent the loss of nutrients from soil erosion (Hoorman, 2016).

Traditionally, the numerous species of bacteria responsible for these three tasks are divided into five functional groups: autotrophs, decomposers, mutualists, pathogens, and chemolithotrophs. The first of these, the autotrophic bacteria, are self-feeding organisms that convert soil chemicals directly into energy for their own use; while the second group, the decomposers, convert soil chemicals into organic matter and simple carbon compounds. The mutualists form associations with plants, helping them to absorb the nutrients the plants need for their survival, and though the pathogenic bacteria cause disease, they also help regulate the population sizes of the other soil inhabitants. Finally, the chemolithotrophs are chemical and rock eating bacteria that play critical roles in the various biogeochemical cycles. Together, all

five of the different types of bacteria essentially convert chemical energy found in the soil into organic matter that is useful to the rest of the organisms in the soil food web, and since each species is critical to the health of the ecosystem, diversity is very important; the loss of even one group could have significant negative consequences.

This fact is especially true for those bacterial groups directly involved in the nitrogen cycle. This biogeochemical cycle is the way ecosystems move the essential element nitrogen throughout the atmosphere, biosphere, and geosphere in different chemically bound forms (Harrison, 2003), and without it, life itself would be impossible. The reason why is because the most common form of nitrogen is nitrogen gas, which makes up almost 78% of the Earth's atmosphere. However, the majority of organisms cannot employ this form of nitrogen to use in their metabolic needs (Khan Academy, 2017). Hence, in order for living things to be able to use the abundant supply of nitrogen in the air, it must first be converted into a more "chemically available form" (Harrison, 2003), and the organisms responsible for this process are different groups of soil bacteria (Harrison, 2003).

There are five stages to the nitrogen cycle: nitrogen fixation, nitrogen uptake through organismal growth, nitrogen mineralization through decay, nitrification, and denitrification. First, certain groups of bacteria convert the nitrogen gas in the air into ammonium. Among these groups are both free-living, non-symbiotic bacteria (the majority of nitrogen fixers) as well as symbiotic bacteria that live in root nodules of the host plant (Encyclopædia Britannica, 2017). Together with the decomposers that break down dead organisms, these bacteria provide plants with the ammonium that is one of the forms of nitrogen which plants can use to build their proteins and nucleic acids.

The next stage, nitrogen uptake, is the conversion of ammonium into organic nitrogen in the form of nitrate– the other form plants can use. Called nitrification, this process (Harrison, 2003) happens when any ammonium not absorbed by plant life is first turned into nitrite and then eventually into nitrate. The bacteria that carry out this process are able to gain energy from it, but it requires the presence of oxygen (Harrison, 2003) which means that it can only happen in environments that are oxygen-rich (such as soils with good structure and water uptake– environments that tend to be bacteria rich in the first place). These nitrifying bacteria are normally eaten in turn by protozoa who cause the release of this nitrate for the plants to use, and in fact, 80% of the nitrogen in plants comes from the bacteria-eating protozoa.

The final stage of the nitrogen cycle is denitrification, and during it, denitrifying bacteria transform oxidized types of nitrogen, including nitrate and nitrite, back into nitrogen gas that then returns to the atmosphere (Harrison, 2003), and the whole cycle repeats. However, anaerobic bacteria can do this under swampy or waterlogged conditions, where there is less oxygen, and can reduce the total amount of fixed nitrogen by around 50% (Encyclopædia Britannica, 2017).

The reason the nitrogen cycle is so critical is because the element nitrogen is an essential component of RNA, DNA, and proteins. These three chemicals are the biological molecules most responsible for the chemical reactions which cause the four tasks of cells to happen (homeostasis, reproduction, synthesis, and transformation of energy), and therefore, they are necessary for all organisms to live and grow (Harrison, 2003, Thomas, 2017). That is why, without the nitrogen cycle and the bacteria that complete it, no ecosystem could function (Harrison, 2003).

Bacteria, though, do more than simply cycle the nitrogen; they are themselves also a vital source of other nutrients for organisms throughout the soil food web. As the largest group of organisms in the soil, they are at the bottom of the food chain for the community of species that live all or part of their lives in the soil, (Kirk, 2013 and Ingham, 1996), and there are many other organisms that consume them, including simple roundworms, shredders (arthropods), earthworms, mites, amoebae, flagellates, and ciliates (Ingham, 1996 and Kirk, 2013). These creatures that eat the bacteria are usually consumed in turn by a secondary consumer, passing on the original nutrients from the bacteria, and as a result, the necessary nutrients for survival for all the soil's inhabitants are able to flow throughout the soil food web (Ingham, 1996).

However, this flow of chemical energy is a fragile one, and the soil food web can easily become disrupted by the presence of artificial substance. One such substance are the pesticides people use in farming and gardening, and as pesticide use becomes more frequent, it is important to consider the effect of these compounds on the life of the soil.

Traditionally pesticides are used to kill unwanted creatures that could possibly damage plant life in a particular area, and in 2007, the world used 5.2 billion pounds of pesticide, of which the United States contributed 20% (Plumer 2013). Furthermore, while there are many different types of pesticides, the six main categories are: herbicides, insecticides, larvicides, bactericides, fungicides, and rodenticides (Australian Government Department of Health 2010).

The most popular insecticide brand in the world is the Bayer Advanced brand, and the active ingredient in this insecticide is a 0.75% concentration of cyfluthrin. (Bayer Advanced 2017). This chemical was first used in the United States in 1987 and is now the most common active ingredient in pesticides. Cyfluthrin is toxic to both insects and other animals such as humans, and it has a particularly devastating effect on bees and fish. When an insect comes into

contact with cyfluthrin, it binds to their nerve cells, which causes over-stimulation to their nervous system. This impairs their essential activities, leading to death. Cyfluthrin is classified as a synthetic pyrethroid, and like many in that classification, the toxicity increases with lower temperatures. Depending on the amount of cyfluthrin that they are in contact with and the duration of exposure, the end results may be chronic (Cox, C. 1994).

Because pesticides are killing a large variety of organisms, they may also be killing bacteria in the soil. This could disrupt the passing of nutrients and organic matter between organisms; hence, the application of pesticides may be negatively impacting the environment through decreasing bacterial diversity. Therefore, in our Soil Ecology experiment, we will be testing the effects of the Bayer Advanced insecticide on the diversity of bacteria in the environment. We will be testing the effect of their popular product, Bayer Advanced Insect Killer (Lawn), on the diversity of bacteria in the environment. Diversity is critical because different species of bacteria play essential roles in maintaining a stable environment. Bacteria also play a critical role in the soil food web, and if the diversity levels fluctuate, it may have a pernicious effect on the future ecosystem. In our experiment, we will strive to learn the human impact of insecticides on the bacterial diversity in the environment. We think that the insecticides will cause a decrease in bacterial diversity in the environment.

Bacteria Diversity Lab Procedure

I. Problem: Do insecticides decrease the amount of diversity of bacteria in the soil?

II. Hypothesis: Insecticides decrease the amount of diversity of bacteria in the soil.

III. Procedure:

a. Independent Variable: Addition of the insecticide to a soil plot.

b. Dependent Variable: The density (Number of bacteria per cubic centimeter) and proportion of different bacteria species in the soil samples.

c. Negative Control: The addition of only water to a soil plot.

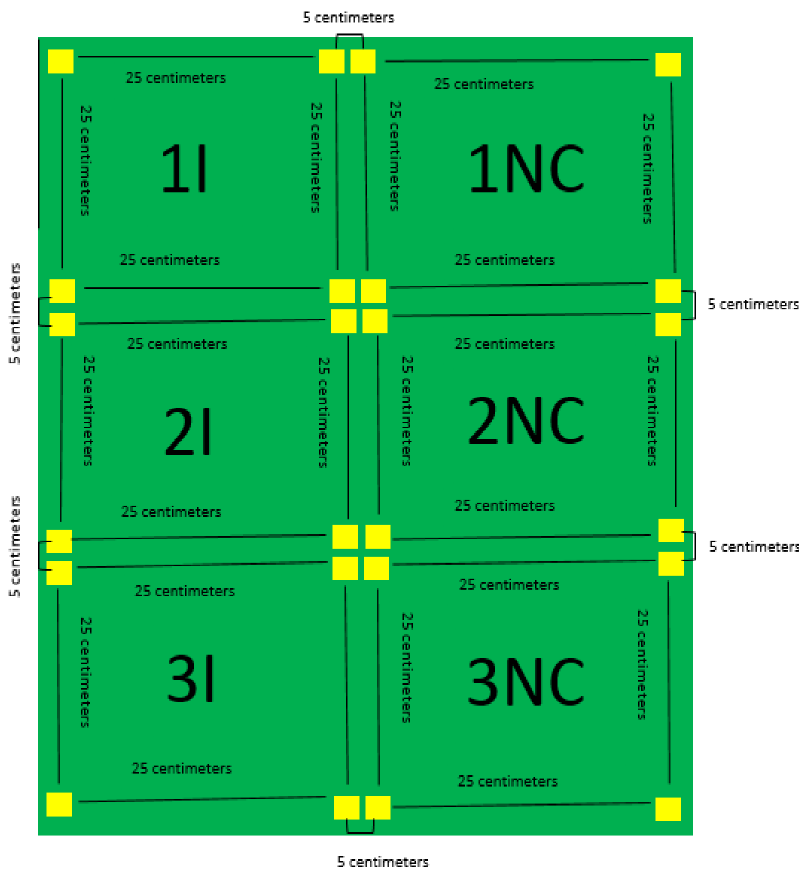
d. Positive Control: The samples of soil that are collected before the application of the Independent Variable.

e. Controlled Variables: Vegetation in environment, core extractor used, sterile water source, amount of time allowed for insecticide to work, amount of insecticide used, size of plot, brand of insecticide, concentration of Cyfluthrin in insecticide, type and amount of food used for growing bacteria, amount of chemicals used in dilution process, amount of time for colonies to cultivate, amount of soil used in dilution process, location of soil tested, distance between plots, distance between spray bottle and ground when spraying, amount of soil collected, size of culture tubes used, amount of sterile water used, location of environment, when (same day, same time of day) soil is extracted, When soil samples are diluted (same day, same time of day) temperature the culture colonies are growing in, how much of the soil dilution is plated, which dilutions are plated, how far each sample is diluted,

f. Steps:

1. Go to North 39° 21.394’ and West 076° 38.112’.

2. Using a black Sharpie marker, label 4 little yellow flags "1 I", representing the first plot of land with insecticide. Label 4 more little yellow flags with "1 N.C.", representing the first plot of land without the insecticide, being the negative control.
3. Repeat step 2 labeling the little yellow flags "2 I" and "2 N.C.", representing the second plot of land
4. Repeat step 2 labeling the little yellow flags "3 I" and "3 N.C.", representing the third plot of land
5. Plot the area based on the diagram below



6. Steps 6 and 7 should have all soil sample columns taken on the same day, at the same time. Use a Soil Core sampler that has a 2 centimeter diameter to extract 15 centimeters deep of

soil from three separate locations on plot 1 and place each one in its own separate plastic bags labeled “1 I PC” (for positive control) with sample 1, 2, and 3 for each bag respectively.

7. Repeat step 6 for plots “1 N.C.”, “2 I”,” 2 N.C.”, “3 I”,” 3 N.C.” in individual plastic bags labeled according to their number with a “PC” (for positive control) and sample 1, 2, 3 for each sample bag.

8. All soil sample combining should happen on the same day at the same time. Take the three soil samples from “1 I PC” and pour all three bags into one bag. Using your hand, grind and combine the soil together until a smooth, wet sand-like texture is achieved. Repeat this process for “1 N.C. PC”, “2 I PC”, “2 N.C. PC”, “3 I PC”, and “3 N.C. PC” at the same time, on the same day as all of the serial dilutions. (NOTE: Be sure to wash hands between bags)

9. Complete Steps 10-23 at the same time, on the same day.

10. Immediately after the soil combining, use a clean, new transfer pipette to add 10 ml of sterile water, to a 15 ml culture tube. Label the tube “10^0 ” and “1 I” using a sharpie (the “I” stands for insecticide).

11. Use the same pipette to add 9 ml of sterile water to a second 15 ml culture tube. Label the tube “10^{-1}” and “1 I” using a sharpie.

12. Repeat step 11 three more times to two additional 15 ml culture tubes, only label them “1 I 10^{-2},” “1 I 10^{-3}", respectively, using a sharpie.

13. Place 1 cc of the soil sample from plot 1 into the “1 I 10^0”culture tube.

14. Cap the tube and shake vigorously.

15. Using a new clean pipette, remove 1 ml of the soil/water mixture from the “10^0 1 I” tube and place into the “10^{-1} 1 I” tube.

16. Cap and shake vigorously.

17. Using the same pipette in step 15, remove 1 ml of the soil/water mixture from the "10^{-1} 1 I" tube and place into the "10^{-2} 1 I" tube.

18. Cap and shake vigorously.

19. Using the same pipette in step 15, remove 1 ml of the soil/water mixture from the "10^{-2} 1 I" tube and place into the "10^{-3}1 I" tube.

20. Cap and shake vigorously.

21. There should now have a total of four culture tubes.

22. Plate 100 µl samples from each of the "10^{-2} 1 I" tube and "10^{-3}1 I" onto their own separate 3M Petrifilm™ Aerobic Count Plates, and label them correspondingly with a sharpie.

23. Repeat steps 9-22 with the soil samples from the bags labeled "1 N.C. PC", "2 I PC" 2 N.C. PC", "3 I PC", 3 N.C. PC".

24. Allow all the samples to grow for the same 72 hour time period.

25. Determine the amounts of different kinds of Bacteria present on the 10^{-3} agar plate, by distinguishing different "types". The "types" are determined based on the different shape, color, size, shade, etc. Type 1 are medium sized, dark red/purple colonies, Type 2 are medium sized, bright red (lighter than type 1) colonies, Type 3 are very small, pale red colonies, Type 4 are very small, dark red colonies, Type 5 are very large, bright red colonies, Type 6 are very large, dark red colonies, and Type 7 are very large gray colonies. Then check the 10^{-2} agar plate for any bacteria types that are different from the bacteria colonies on agar plate 10^{-3}. Examine the Positive controls first beginning with "1 I PC", then "1 N.C. PC" and continuing with the other samples, and then the data. Use a magnifying glass and a pencil to point at and count the bacteria. If there are an excessive amount of a certain type of bacteria, count the amount of that

type of colony on a single square on the agar plate, then multiply that by the number of squares that have that type of bacteria present. Apply the number to this equation:

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

26. Calculate the average numbers for bacteria types 1-7 of the insecticide plots and the negative control plots (for positive control). Make a pie chart for each section of data, there should be two--insecticide positive control and negative control positive control.

27. Add the information to the “Before Experiment is conducted” section of the data table

28. Using proper safety precautions and a sterile serological pipette, mix 5.4 milliliters of Bayer Advanced Insect Killer (for lawns) (with a 0.75% concentration of cyfluthrin) into 231.6 milliliters of water. This must be done on the day that it will be applied to the plots.

29. On the same day, at the same time, use a new sterile serological pipette to add 79 milliliters to the center of plot “1 NC”. Repeat this for “2 NC”, and “3 NC” respectively, from a distance of 3-5 centimeters off the ground.

30. Using a new sterile serological pipette, add 79 milliliters of the insecticide solution to each of the centers of plots “1 I”, “2 I” and “3 I” from a distance of 3-5 centimeters off the ground.

31. Wait 48 hours for the soil to absorb the liquids.

32. Steps 32-33 for all of the soil samples must be taken on the same day, at the same time. After 24 hours, use a Soil Core sampler that has a 2-centimeter diameter to extract 30 centimeters of soil from each of the three locations on plot “1 I” and place it in three separate plastic bags labeled “1 IA” (for After the experiment) and 1, 2, and 3 respectively for the sample numbers.

33. Repeat step 32 for plots “1 N.C.A”, “2 IA”,” 2 N.C.A”, “3 IA”,” 3 N.C.A” (in individual plastic bags labeled according to their number with an “A” for After the Experiment). This must be done on the same day and same time as step 32.

34. Repeat steps 8-26 with the new soil samples from after the insecticide applied. All of the dilutions must occur on the same day, at the same time. Repeat this process for soil samples “1 N.C.A”, “2 IA”,” 2 N.C.A”, “3 IA”, and “3 N.C.A” at the same time.

35. Add the bacterial diversity levels to the “After Experiment is conducted” section of the data table.

IV. Data and Analysis

Data Table:

The key below is the phenotype descriptor used to identify and categorize the types of bacteria in this experiment. The key and numerical system applies to all data tables and graphs below.

Type 1- Medium sized, dark red/purple

Type 2- Medium sized, bright red (lighter than type 1)

Type 3- Very small, pale red

Type 4- Very small, dark red

Type 5- Very large, bright red

Type 6- Very large, dark red

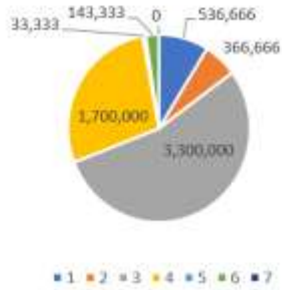
Type 7- Very large, Gray

Diversity of Soil Bacteria Before Insecticide is Added (Density of each type: # / cm^3)								
	Plots with Insecticides Applied				Plots with only Water Applied (negative control)			
Types:	Plot 1 Positive control	Plot 2 Positive control	Plot 3 Positive control	Averages Positive control:	Plot 1 positive control	Plot 2 positive control	Plot 3 positive control	Averages negative controls:
Type 1	400,000	50,000	200,000	216,666	1,500,000	100,000	10,000	536,666
Type 2	500,000	40,000	200,000	246,666	100,000	1,000,000	0	366,666
Type 3	500,000	70,000	300,000	290,000	1,400,000	100,000	8,400,000	3,300,000
Type 4	1,300,000	40,000	700,000	680,000	2,300,000	400,000	2,400,000	1,700,000
Type 5	200,000	10,000	100,000	103,333	0	100,000	0	33,333
Type 6	200,000	20,000	100,000	106,666	400,000	30,000	0	143,333
Type 7	100,000	0	10,000	36,666	0	0	0	0

Diversity of Soil Bacteria After Insecticide is Added (Density of each type: # / cm^3)								
Plots with Insecticide Added					Plots with only Water Applied (negative control)			
Types:	Plot 1	Plot 2	Plot 3	Averages for insecticide	Plot 1	Plot 2	Plot 3	Averages for negative control
Type 1	400,000	500,000	600,000	500,000	120,000	200,000	900,000	406,667
Type 2	300,000	600,000	300,000	400,000	300,000	300,000	300,000	300,000
Type 3	900,000	3,600,000	1,300,000	1,933,333	100,000	700,000	12,600,000	4,466,667
Type 4	11,600,000	31,500,000	5,200,000	16,100,000	75,600,000	5,100,000	1,500,000	27,400,000
Type 5	100,000	0	200,000	100,000	0	300,000	0	100,000
Type 6	100,000	0	300,000	133,333	10,000	100,000	200,000	103,333
Type 7	0	100,000	0	33,333	10,000	100,000	0	36,667

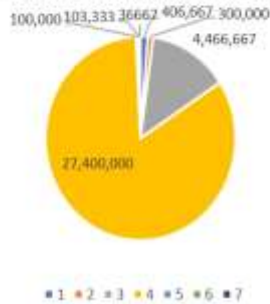
Graphs:

Diversity of Soil Bacteria Before Water is Added (Density of each type: #/cm^3)

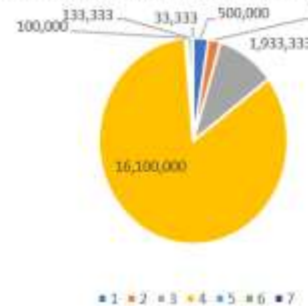


Diversity of Soil Bacteria Before Insecticide is Added (Density of each type: # / cm^3)

Diversity of Soil Bacteria After Water is Added (Density of each type: # / cm^3)



Diversity of Soil Bacteria After Insecticide is Added (Density of each type: # / cm^3)



V. Conclusion

Our hypothesis that insecticides decrease the amount of diversity of bacteria in the soil was incorrect. It was incorrect because the diversity of the bacteria decreased drastically in both the plots with only water applied and the plots with the insecticide applied. In the positive control data, it is shown that both the negative control plots and the insecticide plots have a lot of diversity because one or two types of bacteria are not dominating the rest. Type four (very small, dark red) and type three (very small, pale red) have the most dense bacterial populations in both, but they are not overwhelming the others. However, in the data from both the negative control and the insecticide plots in the actual experiment, type four and type three of the bacteria have a

much denser population than they did in the positive controls, therefore, they overwhelm the other types of bacteria; this shows that the diversity has decreased extremely because one or two types of bacteria have much higher populations than the other bacteria. Type four bacteria makes up 54% of the total bacteria in the positive control before water was added and type three bacteria takes up 40% of the total bacteria in the positive control before insecticide was added. After the insecticide and the water were added, type four took up 83% of all bacteria for both sections. The number of type four bacteria increased by 2,267% for the insecticide plots and 1,511% for the negative control plots from the positive control data to the data found after the experiment. The number of type three bacteria also increased; it increased by 566% for the insecticide plots and 35% for the negative controls plots. All of the other types of bacteria either had a small decrease or a less notable increase. The amount of type one (medium sized, red/purple) bacteria increased by 130% in the insecticide plots, however, this is not very significant because it is not a large number before or after the increase. It decreased by 24% in the negative control plots. Type two (medium sized, bright red) increased by 62% in the insecticide plots (but is it not a very significant number because it is relatively small) and decreased by 18% in the negative control plots. Type five (very large, bright red) decreased by 3% in the insecticide plots and increased by 200% in the negative control plots (but, again, this increase is not a very significant number because it is relatively small before and after the increase). Type six (very large, dark red) increased by 25% in the insecticide plots and decreased by 38% in the negative control plots. Lastly, type seven (very large, gray) decreased by 9% in the insecticide plots and increased by 3,666,700% in the negative control plots (it went from 0 to 36,667, so the percentage increase is very high). Overall, the sheer number of type three and type four bacteria in the data after the experiment shows that the diversity decreased because there

was an enormous amount of two types of bacteria, and the other bacteria were in much smaller quantities (than type 3 or type 4), unlike the positive control data.

The fact that types three and four of the bacteria had the largest populations in both locations, tells us that the resources they need to complete their functions must have entered into the environments of all plots. The spike in their unique resources allowed them to reproduce at much faster rates than the other bacteria groups, thus making their populations extremely large. There was a decomposing tree stump (and its roots) around our plots. There was an excessive amount of rain between the time our positive control samples were taken and the time our insecticide and negative control samples were taken. When it rained, the organisms that decompose wood started working at a faster rate, while doing this they released certain chemicals into the ecosystem. The type of organisms that decompose wood are called actinomycetes, which are a “higher-form bacteria similar to fungi and molds” (University of Illinois, 2017 and Titus and Pereira, 2009). When they decompose wood and other materials such as cellulose, they release carbon, nitrogen, and ammonia (University of Illinois, 2017). We suspect that the two dominant types of bacteria in our experiment use these chemicals, released by the actinomycetes, as a food source, therefore, causing their reproduction rates to increase extremely.

In the future, the first experiment we would conduct would be to find out the types of bacteria that types three and four are. After finding the types out, we would manipulate the amount of carbon in the ecosystem and see the effects it had on the size of the population of type three. Then, we would repeat the same experiment with nitrogen and ammonia on type three. After manipulating all three chemicals for type three, we would repeat all experiments for type four of the bacteria. If the populations in a particular test spiked, we would conclude that the

bacteria types used that chemical as a food source, which is why their populations in our experiment increased so much in such a short amount of time.

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