Genome sequencing of bacteria from soil samples around Hampden-Sydney College to judge soil health levels

Brennan M. Vaught ’21, and Michael J. Wolyniak

Department of Biology, Hampden-Sydney College, Hampden-Sydney, VA 23943

INTRODUCTION

Bacterial genera diversity is an important indicator of soil health: the more diverse the genera of the bacteria colonies present, the healthier the soil tends to be.1 Diversity is also important because it allows for the cycling of “major elements”—carbon, nitrogen, and phosphorus—which maintains healthy soil. 1 To start, this research project was focused solely on sequencing the 16S rRNA section of different bacteria colonies in order to judge the health of the soil based on what bacteria were present. However, in order to adequately assess soil health this way, thousands of sequences would have had to have been ran per location. That simply was not possible for both monetary and time reasons. So, the project was reformed to look at other indicators of soil health, not just the diversity of bacterial communities—however the diversity of the communities was the heaviest weighted indicator. The other considered indicators were soil pH and levels of nitrogen, phosphorus, and potassium. These indicators were chosen because they can be tested with simple soil test kits purchasable from Lowes or Wal-Mart.

The best indicator of soil health is bacteria colony diversity; healthy soils exhibited higher microbial diversity than the bacterial wilt infected soils.1 A higher diversity in bacteria colonies leads the pH of the soil to be more neutral, the best possible pH for most plants and bacteria. Soil pH is the second-best indicator of soil health because a neutral soil pH (a pH close to 7.0) provides both plants and bacteria with the most favorable environment. At very acid or alkaline pH levels, organic matter mineralization is slowed down or stopped because of poor microbial activity linked to bacteria.2

Soil pH is also related to the amount of nitrogen, phosphorus, and potassium available within the soil. When soil pH is neutral, there is usually a high composition of nitrogen, phosphorus, and potassium. However, when soil pH levels are poor, there is a deficiency of nutrients, a lower level of microbial activity, and thus less plants growing in the soil. Furthermore, diseases can be more prominent in alkaline or acidic soils. For example, take-all, a plant disease, is caused by the fungus Gaeumannomyces graminis, which is favored by alkaline pH and infects wheat, barley, rye, and several grasses.2

It was predicted that if the soil had a higher diversity of the bacteria growing, then there would be a more neutral pH, as opposed to an alkaline or acidic pH, and, thus, higher levels of nitrogen, phosphorus, and potassium.

MATERIALS AND METHODS

Making of the Agar plates

The Luria Broth (LB) plates were made by adding 10g of granulated Luria Broth to 500 mL of EPURE water. The solution was then autoclaved and set to cool while stirring to prevent the agar from solidifying. After the solution cooled down, the solution was poured into clean plates and set to solidify for 3 days. Tryptic Soy Agar (TSA) plates were prepared in the same way; however, the starting material was 1.15 g of Bacto Tryptic Sor Broth. The procedure for the Martin Agars was also like that of the previous two but the four starting materials were 11.5 g peptone, 0.5 g starch, 2.5 g Sodium Chloride, and 6.5 g agar.

Soil Sample Selection

The location from which the soil samples were collected was marked with a pin on Google Maps. While wearing gloves, a circle hole was dug/cut with a knife and the soil was scooped up with a plastic spoon. When enough soil was collected, the spoon was placed in the bag, the bag was labeled with name of location and latitude and longitude, and the knife was cleaned. This procedure was completed for each of the 4 locations.

Soil Dilutions and Bacterial Upbringing

Soil Dilutions were prepared following the exact procedure outlined by Jove. Each plate was separated into 3 parts, one for the “C” dilution, one for the “D” dilution, and one for the “E” dilution. Then, the plates were labeled with the following: plate type, soil location, date, temperature of storage, and initials of researcher. A volume of 0.1 mL of the “C” dilution was then pipetted into its region of the plate and spread using a glass spreader. The spreader was then sterilized, and dilutions “D” and “E” were pipetted into their regions using the same technique as dilution “C.” The plates were then taken to their respective temperatures for growth (1-3 days).

Bacterial Diversity Ranking

When the soil diversity of the locations was being ranked, it was based solely on what was seen, not from the sequencing results because not all colonies could be sequenced due to money and time available. The plates were all compared side-by-side and at the same time in growth (5 days).

Cultured Bacterial Specimens
Bacteria were cultured from the soil dilution aseptically using an inoculating loop and a Bunsen burner. A specific bacterial colony was chosen, and the inoculating loop was passed through the Bunsen burner, and allowed to cool before making contact with the chosen bacteria colony. Then the bacteria colony was scooped up with the inoculating loop and transferred to a clean plate of the same type. The new plate was then labeled with plate type, soil location, date, temperature of storage, initials of researcher, and specimen number. This was repeated for each specimen that was cultured.

DNA Extraction, PCR, and Gel Electrophoresis
DNA extraction was performed using the Quiagen DNeasy UltraClean Microbial Kit. PCR was conducted on each of the extracted DNA sequences to separate the 16S rRNA sequence and to amplify said sequence. The PCR went through 35 cycles to ensure that the sequence was plentiful. Gel electrophoresis was conducted to see if the DNA from the microbes was extracted properly. The microbial DNA was compared against a 100 bp ladder and loaded with EZ Vision to ensure that the DNA could be seen.

DNA Clean Up and Sequencing
The DNA from the microbes was cleaned up in accordance with the "PCR Clean Up Protocol" section of the IBI Scientific Gel/PCR DNA Fragments Extraction Kit. One step was modified in order for the samples to be in proper form for the sequencing center in Virginia Tech: in step 3 of the protocol, instead of using the Elution Buffer, sterile water was used. The DNA was then prepared and loaded into PCR tubes as per Virginia Tech's Sequencing Center's guidelines for submission. Then the DNA samples were packed in a cooler and shipped to the Sequencing Center via UPS overnight shipping. This was to ensure that the DNA did not denature.

Soil Testing
Two soil test kits were used. First was the HoldAll Soil Test Kit, purchased from Lowes, and the second was the Rapitest Soil Test Kit, purchased from Wal-Mart. The procedures for these kits were included in the kits and were followed exactly.

RESULTS

It was observed that location C had the most diverse bacteria colonies, followed by Location A. Location B was third in terms of being diverse and Location D appeared to have the least diverse bacteria colonies. Pictures of plates from which samples were taken are on pages 8-11. Below is a representative picture of plates with bacteria growth. This image is called "representative" because it shows a plate from each location that is representative of the bacteria colony diversity.

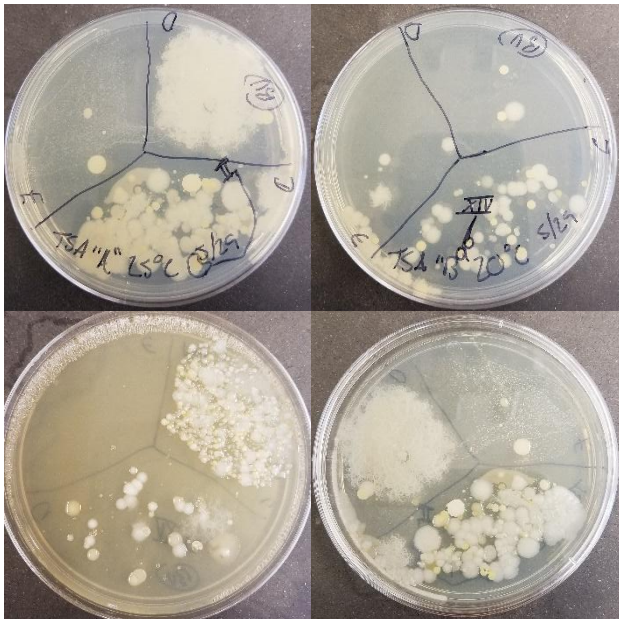

Figure 1- Representative Pictures of the Bacteria Growth on the Plates (Left to Right; Location A, B, C, D)

Location A was near the Hampden-Sydney water tower (37°14'11.5"N, 78°27'38.9"W). This soil was a red clay-silty type of soil. The average pH over the 4 different trials was 5.88, which points towards an acidic soil. The nitrogen test for location A came back nonapplicable; the phosphorus gave an average score of 2.00. The potassium test for location A had an average of 2.33. The total score for location A was 10.21, giving it a last place ranking.

There were six specimens taken form the soil at Location A. Of these six, two were sequenced to be of the *Bacillus* genus, one of *Chromobacterium*, one of *Pseudomonas*, one of *Rahnella*, and one was inconclusive. This shows fairly diverse bacteria colonies since there was four different genera within six sequences.

Location B was located near the pond at the Tiger Rec center (37°14'39.1"N, 78°27'18.5"W). The soil here was a loamier soil, a darker brown color than Location A and D. Location B gave a slightly higher average pH with a 5.94. Like location A, the nitrogen test for location B came back nonapplicable; however, the phosphorus test gave an average of 2.50. The potassium test for location B had an average of 2.33. The total score for location B was 10.77, placing it third. Location B yielded five specimens, one *Pseudomonas* genus, one *Ralstonia*, one *Lysinibacillus*, one *Microbacterium*, and one inconclusive sequence. Since four of the five sequences gave four different genera of bacteria, it was concluded based solely on the sequencing that Location B had more diverse bacterial colonies than Location A but less diverse than Location C.

Location C was right beside Fulton field, the football field (37°14'40.5"N, 78°27'31.2"W). The soil here was the darkest of all the location and was a balance between clay, loamy, and silty. Location C's soil pH was neutral at 6.88. The nitrogen test came back nonapplicable, and the phosphorus test yielded a perfect 4.0 score. The potassium test for location C had an average score of 2.67. The total score for location C was 13.54, the highest score recorded.Like Location A, Location C had six specimen bacteria colonies sequenced. There were five different genera that came from these six sequences: *Ralstonia*, *Bacillus*, *Pseudomonas*, *Lysinibacillus*, and *Rahnella*. Since there were five different genera out of six sequences, Location C was seen to have the highest diversity of bacterial colonies out of all four soil locations.

Location D was in front of Gilmer Hall, underneath of the tree in the right, in a bare spot (37°14'28.1"N, 78°27'50.1"W). This location was chosen because it was a different type of environment than any of the other locations. Location D had an average pH of 5.91 and a nitrogen test of nonapplicable. The phosphorus score for location D was a 2.50 and the potassium test gave a 3.00. The total score for location D was 11.41, the second highest score. Location D had a very high concentration of the *Bacillus* genus. Four out of the five sequences ran from Location D were *Bacillus*, with one being *Pseudomonas*. It was concluded that Location D had the least diverse soil based on the sequencing results.

The data above can be found on pages in the appendix below. The sequencing results can also be found in the appendix. Included in the sequencing results table is the specimen number, specimen isolation site, and the sequencing result. Below is a picture of the sequence and chromatogram of Specimen 6.

Table 1- Soil Sample Locations

Location	Description	Latitude	Longitude
A	Near the water tower on campus	37°14'11.5"N	78°27'38.9"W
B	Close to the pond by Tiger Rec	37°14'39.1"N	78°27'18.5"W
C	On the southside of Fulton field	37°14'40.5"N	78°27'31.2"W
D	In front of Gilmer Hall	37°14'28.1"N	78°27'50.1"W

CGTAGCGCCGCTTACCTGCAGTCGACGGCAGCATGATCTACCTTGCTAGATTGATGG
CGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCCTGTATGGGGGGATAACT
AGTCAAAAGATTAGCTAATACCGCATACCACCTGAGGGTGAAAGTGGGGGACCGCA
GGGCCTCCTGCTATAGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGAGGTAAAGGC
TCACCAAGGCGACGATCACTATCTGGTCTGAAAGGACGATCAGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGC
GAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCA
CTTTTGTCCGGAAAGAAATGGATCTGGTTAATACCTGGGGTCGATGACGGTACCGGA
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CAGACGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGAAGGAAT
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GATGATGTGGATTAAGTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCTA
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CATGGCTGTCGGCAGCTCGTGTCGTGAGATGTTGAGATAAGTCCCTCAACCAGCGCA
ACCTTGTCTCTAGTTGCTACGATAGGGCACTCTAGATAGACTGCCGATGACGACCGG
CAGGAACGAGCGATGACGTCAGTCCTCATGTCCATTATGGATAGGGCTTCACACGTC
ATACAATGATGCATTCAGAGGGTAGGCTAGCAGCGAGTGTGGAACTATCCGATAAA
TCGCATCGTAGTACCGGATCGTAGCTGCGAACTCGACTACTTGAGCCTGGCATTCGC
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ATATCCTCGCAGGTCAAG

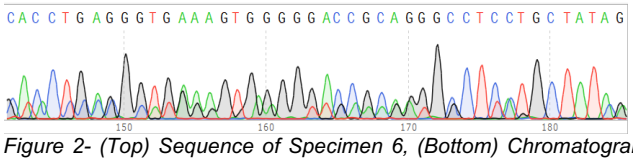

Figure 2- (Top) Sequence of Specimen 6, (Bottom) Chromatogram of Specimen 6. This specimen was 1430 bases long. The top picture shows this exact sequence and the bottom picture is a segment of the Chromatogram from this sequence. The reason that there are different colors is because each color coordinates to a different nucleotide base. In sequencing, when the correct nucleotide is attached, a light is emitted, a detector picks up the wavelength of light and sends a signal to computer, which records what the corresponding nucleotide was.

DISCUSSION

The hypothesis was that if there was a higher diversity of bacteria colonies, then the soil at that location would have a more neutral pH, and higher levels of nitrogen, phosphorus, and potassium. For the four locations that were tested, the hypothesis was not accepted. Location C, which had the most diverse bacteria colonies, also had the most neutral pH, a perfect score on the phosphorus test, and the second highest score on the potassium test. As explained earlier, the nitrogen test did not work due to an error within the test that is not known. The error could have been human or a malfunction within the tests.

Location B was the third-most diverse soil. Other than the pH test, in which Location B scored the second-closest to neutral, Location B scored third in both the phosphorus and potassium tests. This supported the hypothesis.

Location A, while it had a large amount of growth, was the least diverse soil in terms of bacteria colonies. Additionally, Location A also placed last in the overall rank. Location A had the most acidic pH test. Along with bacteria diversity and pH, Location A placed last and tied for last in the phosphorus and potassium tests. This did support the hypothesis that lower diversity equaled lower soil health.

The hypothesis was also true for the second-most diverse soil: Location D. Location D had the highest score on the potassium test, and the second-

highest score on the phosphorus test. These high potassium and phosphorus test scores may have been due to the high concentration of the *Bacillus* genus within the soil—3/4 of the sequences ran from Location D gave a *Bacillus* genus. Location D was also underneath of a tree with no grass; this could have also contributed to the higher potassium and phosphorus concentrations and the high *Bacillus* concentration.

CONCLUSION

From looking at the diversity of bacteria colonies within soil from four locations across the campus of Hampden-Sydney College, the following hypothesis was formed: if the bacteria colonies within a soil sample are more diverse, then the healthier the soil is. A higher bacteria diversity also correlated directly with a more neutral pH of the soil, along with higher levels of both phosphorus and potassium. For only two locations—Location C and Location B—was the hypothesis supported; the level of diversity of the bacteria colonies correctly predicted the overall health of the soil when compared to the other locations. For Location A and Location D, the hypothesis was not supported because the level of bacteria colony diversity did not correctly predict the rank of the respective soil location.

The diversity of the bacteria at Locations B, C, and D, left me with the conclusion that these areas had the healthiest soil because these locations had the most average scores on all of the tests. For Location A, the predominance of the Bacillus genus gives higher level of potassium but makes the pH of the soil more acidic.

REFERENCES

[1.] Chourasiya, Dipanti, et al. “Microbial Diversity and Soil Health in Tropical Agroecosystems.” *SpringerLink*, Springer, Dordrecht, 31 Jan. 2018, link.springer.com/chapter/10.1007/978-981-10-7380-9_2.

[2.] Wang, Rui, et. al. “Microbial Community Composition Is Related to Soil Biological and Chemical Properties and Bacterial Wilt Outbreak.” *National Center for Biotechnology Information*, 23 Mar. 2017, www.ncbi.nlm.nih.gov/pmc/articles/PMC5428506/.

[3.] *Soil Quality Indicators: Soil pH*. USDA Natural Resources Conservation Service. April 2011. https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/health/assessment/?cid =stelprdb1237387. Accessed 14 June 2018.

[4.] *Culturing and Enumerating Bacteria from Soil Samples*. Jove, https://www.jove.com/science education/10099/culturing-and-enumerating-bacteria-from-soil-samples. Accessed 11 Mar. 2018

APPENDIX

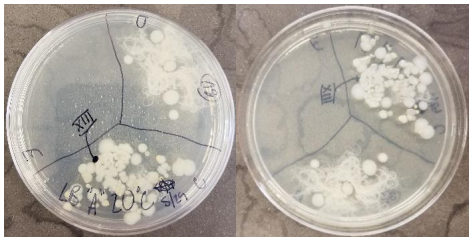

Figure 3- Location A, LB @ 20° C

Figure 4- Location A, LB @ 25° C

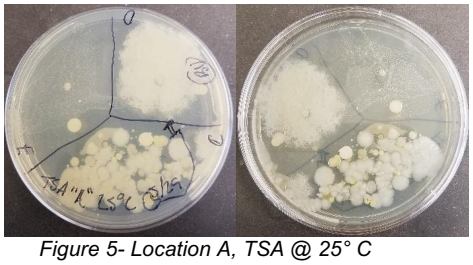

Figure 5- Location A, TSA @ 25° C

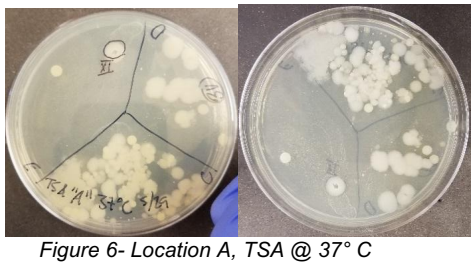

Figure 6- Location A, TSA @ 37° C

Figure 7- Location B, LB @ 25° C

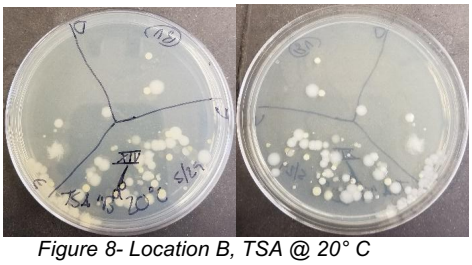

Figure 8- Location B, TSA @ 20° C

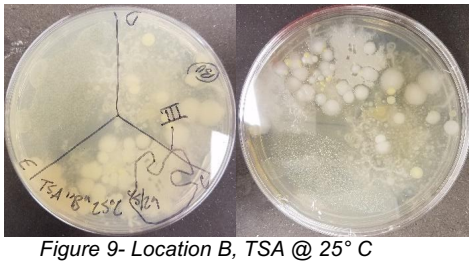

Figure 9- Location B, TSA @ 25° C

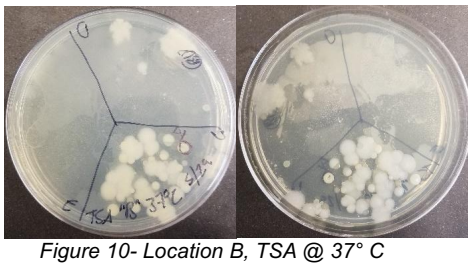

Figure 10- Location B, TSA @ 37° C

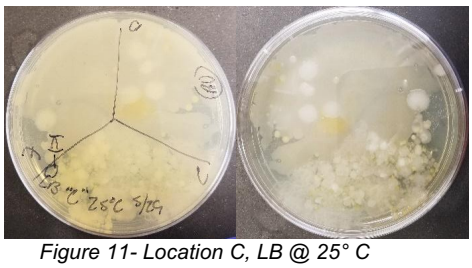

Figure 11- Location C, LB @ 25° C

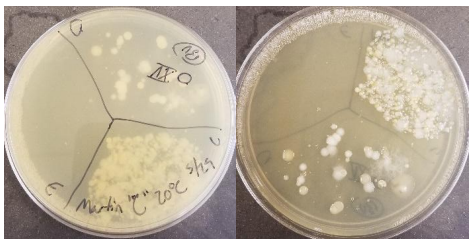

Figure 12- Location C, Martin Agar @ 20° C

Figure 13- Location C, Martin Agar @ 25° C

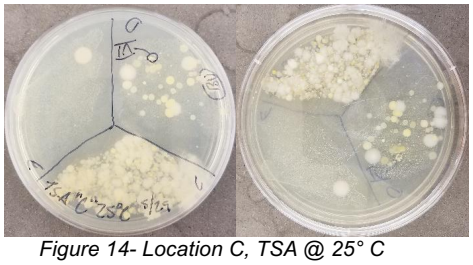

Figure 14- Location C, TSA @ 25° C

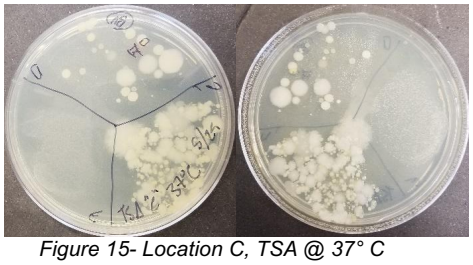

Figure 15- Location C, TSA @ 37° C

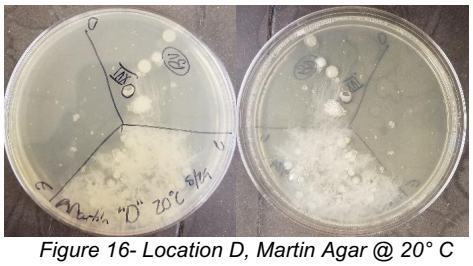

Figure 16- Location D, Martin Agar @ 20° C

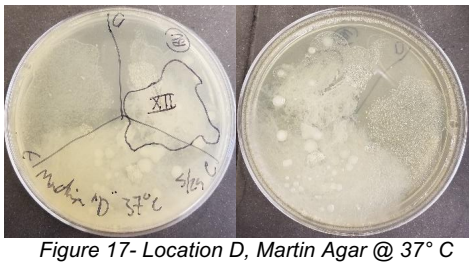

Figure 17- Location D, Martin Agar @ 37° C

Figure 18- Location D, TSA @ 25° C

Table 2- All Data Recorded for Soil Test Kits

Location	Kit Type	pH	Nitrogen	Phosphorus	Potassium
A	HoldAll (Trial 1)	6.00	N/A*	2.00	1.00
B	HoldAll (Trial 1)	7.00	N/A*	1.00	3.00
C	HoldAll (Trial 1)	7.50	N/A*	4.00	3.00
D	HoldAll (Trial 1)	6.50	N/A*	1.00	3.00

A	HoldAll (Trial 2)	6.00	N/A†	N/Aα	3.00
B	HoldAll (Trial 2)	5.50	N/A†	N/Aα	2.00
C	HoldAll (Trial 2)	6.75	N/A†	N/Aα	3.00
D	HoldAll (Trial 2)	5.50	N/A†	N/Aα	3.00
A	HoldAll (Trial 3)	5.75	N/A**	N/Aα	3.00β
B	HoldAll (Trial 3)	5.25	N/A**	N/Aα	3.00β
C	HoldAll (Trial 3)	6.25	N/A**	N/Aα	3.00β
D	HoldAll (Trial 3)	5.00	N/A**	N/Aα	3.00β
A	RapiTest	5.75	N/A††	2.00	3.00
B	RapiTest	6.00	N/A††	4.00	2.00
C	RapiTest	7.00	N/A††	4.00	2.00
D	RapiTest	6.65	N/A††	4.00	3.00

*-Test was a milk-white color
†-Test was clear
**-Test was a yellow-gold color
††-Test was completely clear
α-Dye from test sat in bottom of test tube
β-Was a little dye in the bottom of the tube

For the nitrogen, phosphorus, and potassium tests, the numbers associated indicate the level of each indicator present. According to the kits, there are 4 levels. 1 is very low or insufficient. 2 is low or adequate. 3 is medium or sufficient. 4 is high or surplus.

Table 3- Averages from "Table 1"

Location	pH	Nitrogen	Phosphorus	Potassium
A	5.88	N/A*	2.00	2.33
B	5.94	N/A*	2.50	2.33
C	6.88	N/A*	4.00	2.67
D	5.91	N/A*	2.50	3.00

*Refer to "Table 1" for clarification

Table 3- Averages from "Table 1"

Location	pH	Nitrogen	Phosphorus	Potassium
A	5.88	N/A*	2.00	2.33
B	5.94	N/A*	2.50	2.33
C	6.88	N/A*	4.00	2.67
D	5.91	N/A*	2.50	3.00

*Refer to "Table 1" for clarification

Table 5- Specimen List

Location	Specimen Number	Name
A	1	*Bacillus cereus* or *Bacillus thuringiensis*
A	2	*Bacillus thuringiensis* or *Bacillus cereus* or *Bacillus toyonensis*
A	9	*Chromobacterium aquaticum* or *Chromobacterium rhizoryzae* or Chromobacterium haemolyticum or Chromobacterium violaceum
A	13	*Yersinia frederiksenii* (76%) or *Rahnella aquatilis* (77%)
A	17	*Pseudomonas koreensis*
A	20	*Rahnella aquatilis*
B	3	*Pseudomonas frederiksbergensis*
B	4	No matches above 49%

B	10	*Ralstonia picketti*
B	14	*Lysinibacillus fusiformis* or *Lysinibacillus xylanilyticus*
B	19	*Microbacterium oleivorans*
C	5	*Bacillus oryzaecorticis* or *Bacillus toyonensis* or *Bacillus cereus*
C	6	*Ralstonia picketti*
C	7	*Ralstonia picketti*
C	11	*Pseudomonas rhodesiae* or *Pseudomonas fluorescens*
C	15	*Lysinibacillus xylanilyticus* or *Lysinibacillus pakistanensis* or *Lysinibacillus fusiformis*
C	21	*Rahnella aquatilis*
D	8	*Bacillus mycoides* or *Bacillus psuedomycoides*
D	12	*Bacillus cereus* or *Bacillus toyonensis* or *Bacillus thuringiensis* or *Bacillus anthracis*
D	16	*Bacillus cereus* or *Bacillus thuringiensis* or *Bacillus oryzaecorticis* or *Bacillus toyonensis*
D	18	*Pseudomonas marginalis*
D	22	*Bacillus cereus* or *Bacillus mycoides*