Effects of Agricultural and Recreational Pollution on Microbial Diversity in Prince Edward County

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Introduction

Microbial diversity is heavily influenced by environmental factors and is therefore threatened by agricultural and recreational pollution (Chen et al., 2013; Emani et al., 2014; Ibekwe et al., 2016; Toes et al., 2008). More specifically, the effects of various pH levels, carbon levels, and organic compounds have been reported on. These environmental factors lowered the microbial diversity of water samples more than in sediment samples (Ibekwe et al., 2016).

Heavy metal pollution has been studied in China and Europe and reported to decrease biodiversity and abundance in microbes in river water (Chen et al., 2013; Toes et al., 2008). In China, paddy soil was tested for the availability of organic carbon and heavy metals were found to decrease soil quality and microbial biomass, therefore inhibiting community diversity (Chen et al., 2013). A lower bacterial abundance was also found in coarse sand; however, the researchers could not determine if this was from environmental factors of the heavy metal pollution (Chen et al., 2013). In Europe, metal exposure length and levels to soil were being tested (Toes et al., 2008). This study found that a high microbial diversity is related to the fact that metal-polluted environments usually contain other types of contaminants like petroleum and chlorinated compounds (Toes et al., 2008). The researchers concluded that metal pollution does not lower diversity. The impact of nitrogen fertilizer pollution on microbial diversity has also been tested in soil samples and it was reported that the impact petroleum pollution depended on the amount of toxic organic compounds present in the soil (Emani et al., 2014). Organochlorines have also been tested to show their effects to microbial diversity in soil and were found to slightly bother microbial diversity (Miguel et al., 2014). In each of these studies, DNA was extracted from each sample and examined to determine the amount of biodiversity and abundance (Chen et al., 2013; Emani et al., 2014; Ibekwe et al., 2016; Miguel et al., 2014; Toes et al., 2008). From this, it can be concluded that metal pollution is a cause of decreased microbial diversity, however, toxic organic pollutants such as nitrogen, nitrates, ammonia, etc. are greater contributors to decreased microbial diversity and abundance (Chen et al., 2013; Emani et al., 2014; Ibekwe et al., 2016; Miguel et al., 2014; Toes et al., 2008).

The goal of this experiment is to investigate how agricultural and recreational pollution effect the microbial diversity of water and sediment collected from Buffalo Creek and the Appomattox River. From the research found, we can hypothesize that the more pollution that is found in the gathered samples, the less amount of microbial diversity will be present.

Methods

Environmental Sample Collection

Twelve nutrient agar plates and four sterile conical tubes were labeled for each location: Appomattox River Soil, Appomattox River Water, Buffalo Creek Soil, and Buffalo Creek Water. Soil and water was collected in the same location at each site. Sediment was taken from underneath the water as the soil sample, while water was taken from the top of each water source. Soil was collected from each site when the sterile tube was placed almost to the soil in the water, then the cap was taken off and soil was scooped inside. Water was collected from each site after each tube was submerged and the cap was taken off and filled up. The tubes were brought back to the Environmental Education Center (EEC).

Bacterial Culture

The water and soil samples were cultured on twelve nutrient agar plates. The soil samples were cultured the same way as the water samples because each conical tube had a significant amount of water present after collection. To dilute, 90µl of sterile nutrient broth was placed into a 1:10 and 1:100 labeled tube. 10µl of the sample was placed into the 1:10 tube and was vortexed. 10µl of the 1:10 solution was then transferred to the 1:100 tube and was vortexed. 100µl of each solution (undiluted, 1:10, and 1:100) was placed onto its properly labeled plate and spread using the hockey stick method. The plates were incubated at room temperature (25°C) for 48 hours. The plates were then wrapped with parafilm and placed in the refrigerator. Each plate was observed for bacterial growth every 24 hours. The number of colonies, color, size, shape, and texture was recorded at those times.

DNA Isolation

Two colonies from each sample (Appomattox Soil, Appomattox Water, Buffalo Soil, and Buffalo Water) were selected to isolate DNA from. 300µl of microbead solution was placed into eight microcentrifuge tubes. A pipette tip was used to scrape each colony off the agar plate. The tip was swirled in the solution so the colony would suspend in it. This was repeated for each sample colony. Eight microbead tubes were properly labeled and 300µl of each solution was added to its respective tube. 50µl of solution MD1 was added to each microbead tube. The tubes

were heated to 65°C for 10 minutes. The tubes were then placed horizontally on a vortex for 10 minutes. The tubes were then centrifuged at 10,000 x g for 30 seconds at room temperature. The supernatant of each was transferred to labeled 2mL collection tubes and 100µl of solution MD2 was added to each. The tubes were vortexed for 5 seconds and incubated for 5 minutes at 4°C. The tubes were then centrifuged again for 1 minute in the same conditions as before. The supernatant from each tube was transferred to labeled 2mL collection tubes. 900µl of solution of MD3 was added to each tube and vortexed for 5 seconds. 700µl of each solution were loaded into the spin filter and centrifuged for 30 seconds at the previous conditions. The remaining supernatant was added to the spin filter and centrifuged again for 30 seconds. The excess liquid was discarded and 300µl of solution MD4 was added and centrifuged for 1.5 minutes. The spin filter from each tube was placed into new 2mL collection tubes. 50µl of solution MD5 was added to the filter membrane and was centrifuged for 30 seconds. The spin filters were discarded and the tubes of DNA were stored at -20°C for one week. DNA purity was then measured using the Nanodrop Spectrophotometer. The ratio of absorbance at 260nm and 280nm was used to assess the purity of DNA.

16s rRNA Amplification (PCR)

Eight PCR tubes containing 6µl of DNA was transferred from a 2mL collection tube to a PCR tube. This was repeated for each PCR tube. 44µl of PCR reaction mix was then added to each tube. This contained 1µl of 10 µM Forward Primer, 1µl of 10 µM Reverse Primer, 25µl of One*Taq* 2X Master Mix, and 17µl of Nuclease-free water. The reactions were mixed using the pipette. The PCR tubes were placed in a PCR machine and were thermocycled. The tubes were at 94°C for 30 seconds during initial denaturation. The tubes then went through 30 cycles at 94°C for 30 seconds, 55°C for 45 seconds, and 68°C for 60 seconds. The final extension was for 5 minutes at 68°C. The tubes were held at 4°C until they could be taken out. The primers used to amplify 16S rDNA had the following sequences: forward- 5'GAGTTTGATYMTGGCTC-3' reverse- 5'-NRGYTACCTTGTTACGACTT-3'.

PCR Clean Up

50µl of PCR product and 250µl of DF Buffer was added to a new microfuge tube. The sample was vortexed and 300µl of the mixture was added to a DF Column. This was centrifuged

for 30 seconds and the flow-through was discarded. The DF Column was placed back into the collection tube and 600µl of Wash Buffer was added to the column. After standing for one minute, the mixture was centrifuged for 3 minutes and 30 seconds. The dry DF Column was transferred to a microcentrifuge tube and 25µl of Elution Buffer was added to the tube. After standing for two minutes, the mixture was centrifuged for two minutes to elute the purified DNA.

Restriction Enzyme Digestion

5µl of Loading Dye was added to each tube. Either 5µl of PCR Product or 5µl of *Msp*I Undigestion was added to each tube. After a total of 10µl was in each tube, the solution was mixed by vortexing. The mixtures were then incubated for 45 minutes at 37 degrees Celsius.

Gel Electrophoresis

A 1.5% agarose gel was casted. 10µl of each sample was loaded into each well, alternating between PCR Product and *Msp*I. The lid was placed on the electrophoresis chamber and the electrical leads were connected. The gel developed at 120V for 30 minutes. The gel was placed under a UV camera, analyzed, and pictures were taken.

DNA Sequencing

In each tube, 5µl of the cleaned PCR product, 4µl of the sequencing primer, and 3µl of deionized water was added. The DNA was sent to EurofinsGenomics and sequenced.

DNA Analysis

Each sequence was analyzed with SnapGene Viewer. Each "N" in the sequence was edited whenever appropriate. The changes were saved and BLAST- Targeted Loci was used to identify each prokaryote. The sequence of the prokaryote was compared to the matched BLAST sequence. The gaps in the sequences of the top 10 BLAST matches were recorded. The *Msp*I digestion sites were compared using NEB cutter to the sequences found in the gel. Information about the BLAST match was found and compared to the original bacteria sample and each prokaryote was identified.

Results

The purpose of this experiment was to investigate the relationship between agricultural/recreational pollution and microbial diversity. Eight environmental samples were obtained from the Appomattox River and Buffalo Creek in Prince Edward County, VA. Microbial colonies isolated from all eight samples were identified through BLAST alignment.

Collection Site

Eight samples were taken within a two-hour span on February 8, 2017. Two soil and two water samples each were taken from the Appomattox River and Buffalo Creek (Figure 1). Samples were collected using sterile tubes.

A. 

B. 

C. 

D. 

Figure 1. Collection sites and water samples from each location. Location of collection at the (A) Appomattox River or (B) Buffalo Creek. Water (top) and soil (bottom) samples taken from the (C) Appomattox River or (D) Buffalo Creek.

Environmental Quality

Samples were taken by Hampden-Sydney students on February 21, 2017 at the Appomattox River and Buffalo Creek. These samples were then tested for various components, indicating the quality of the water (Table 1).

	Appomattox River	Buffalo Creek
pH	8.02	12.20
Salinity (ppt)	0.05	64.20
Diss Oxygen (ppm)	10.0	0.1
Phosphate (ppm)	0.70	11.60
Sulfide (ppm)	0.0	0.8
Carbon Dioxide (ppm)	3.0	0.0
Fluoride (ppm)	0.35	4.00
Ammonia (ppm)	0.5	0.0
Total Hardness	40	120

Table 1. Environmental quality results of samples taken at each location.

Microbial Diversity

Each environmental sample was placed on an agar plate as undiluted, 1:10 diluted, and 1:100 diluted. Over the course of one week, many colonies had formed on each agar plate. The plates containing the sample of Appomattox River soil had the most colonies at 24, 48, and 168 hours (Figure 2). The plates of Appomattox River water and Buffalo Creek water had similar amounts of colonies at 24, 48, and 168 hours (Figure 2).

At one week, colony color amounts were counted. The colonies varied in color and were recorded (Figure 3). Many of the colonies on each plate were white. The diversity differed greatly in each site; some had purple colonies as well as red, while others did not. The plate from the Appomattox River water had ≈42 white colonies and ≈20 orange colonies (Figure 4A). The plate containing the sample of Appomattox River soil had ≈81 white colonies, 6 orange colonies, 1 red colony, and 8 purple colonies (Figure 4B). The sample of Buffalo Creek water had ≈62 white colonies, 10 white colonies, and ≈20 purple colonies (Figure 4C). The plates containing the same of Buffalo Creek soil had ≈23 white colonies and 5 orange colonies (Figure 4D).

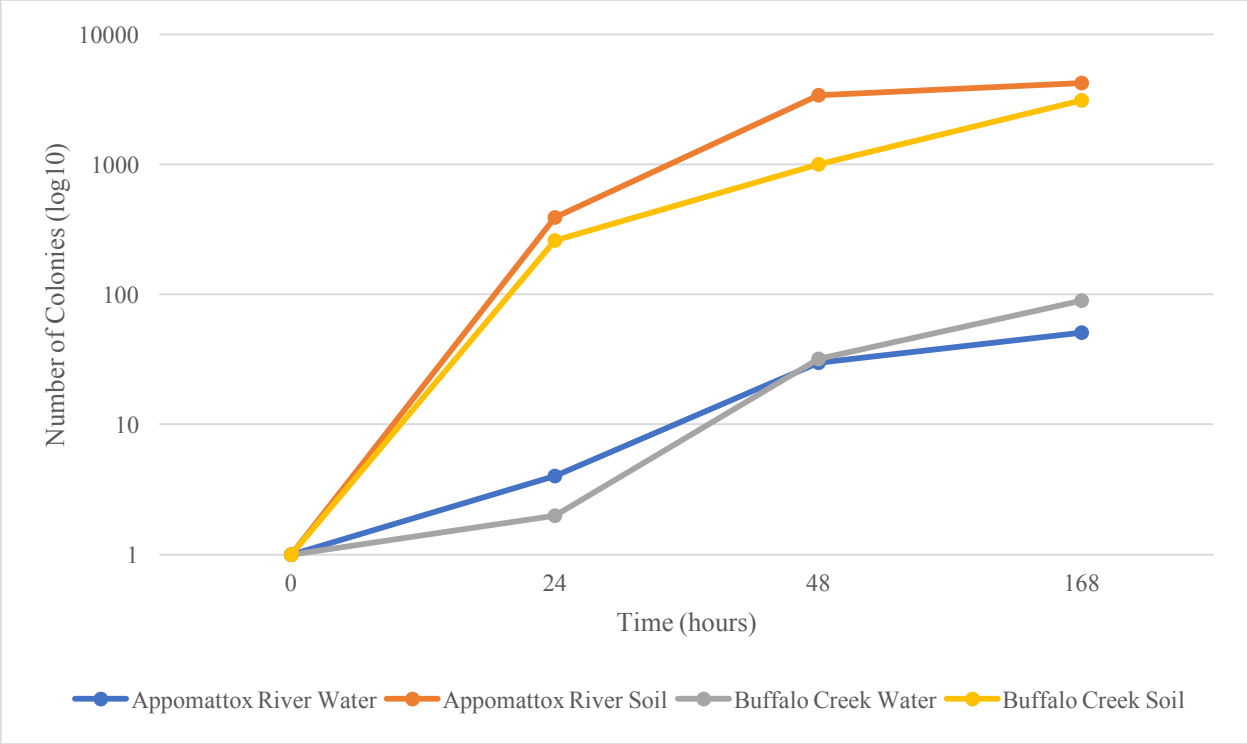


Figure 2. The approximate number of colonies formed after one day, two days, and one week distinguished between sample location. *Graph is shown on a log scale for clarity.

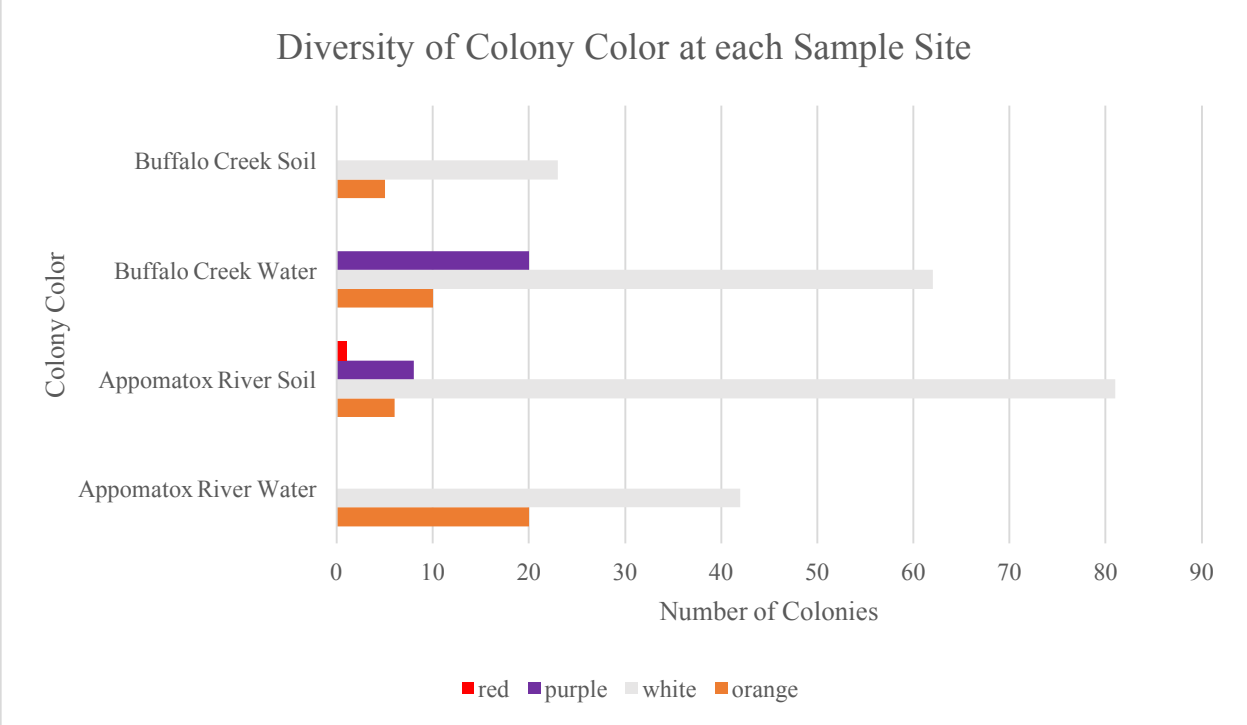
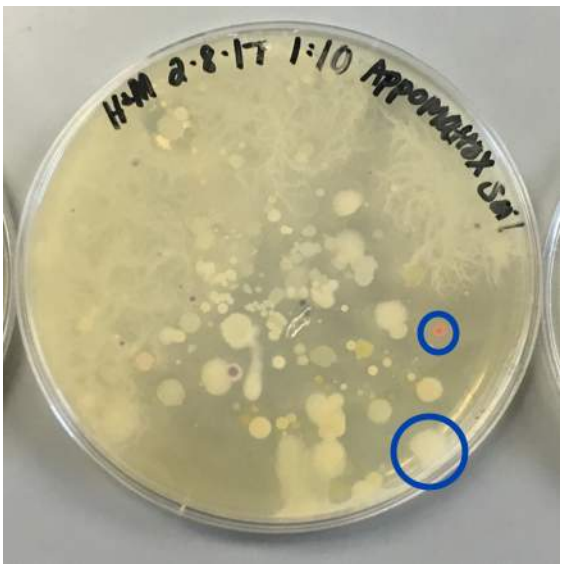


Figure 3. The counts of each color present on the selected agar plates, organized by location of sample site.

A.  B. 

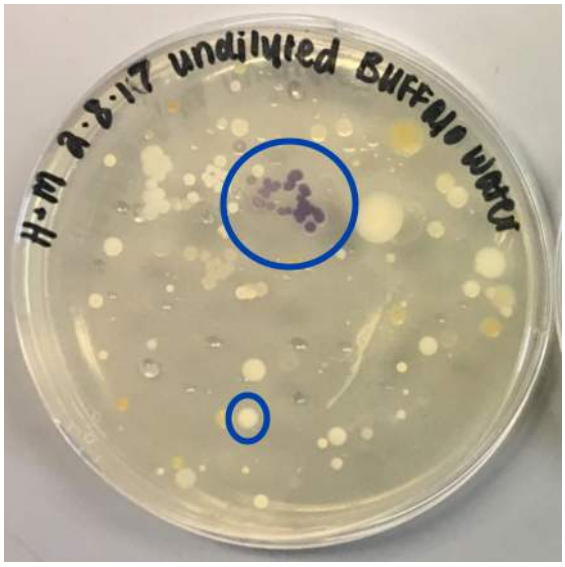
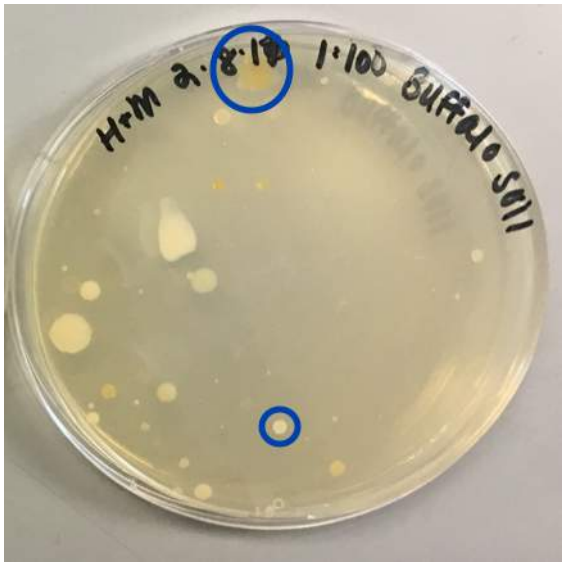
C.  D. 

Figure 4. Colony growth after 1 week from each sample site with selected colonies circled. (A) Colonies present in undiluted sample of Appomattox River water. (B) Colonies present in 1:10 dilution of Appomattox River soil sample. (C) Colonies present in undiluted sample of Buffalo Creek water. (D) Colonies present in 1:100 dilution of Buffalo Creek soil sample.

Amount and Purity of DNA

DNA was obtained by scooping each colony from the agar plates. DNA purity was measured using the Nanodrop Spectrophotometer. The ratio of absorbance at 260nm and 280nm was used to assess the purity of DNA. Samples from the soil from both the Appomattox River and Buffalo Creek had higher ratios of DNA in them (Table 2). A ratio of ≈1.8 is considered "pure". Samples from the soil from both rivers were closer to being "pure" (Table 2).

Location	Sample Number	Concentration (ng/μl)	Purity in 260nm/280nm
Buffalo Creek Soil	1	19.3	1.98
	2	3.9	2.27
Buffalo Creek Water	1	14.7	2.04
	2	11.9	1.85
Appomattox River Soil	1	7.1	1.95
	2	4.9	1.84
Appomattox River Water	1	3.6	1.76
	2	11.7	1.95

Table 2. The amount and purity of DNA isolated from various sampling locations.

Gel Electrophoresis

Each DNA sample went through PCR cleanup and *Msp*I digestion before being developed through Gel Electrophoresis. Each chosen colony was loaded into a 1.5% agarose gel and ran. The gel was developed and analyzed. Bands at 1500bp were brightest in Appomattox soil PCR products, Appomattox water PCR products, and the Buffalo soil 1 PCR product (Figure 5). Dull bands were still produced at 1500bp in Buffalo soil 2 PCR product and Buffalo water PCR products. Dull bands were produced at approximately 500bp in Appomattox soil 1&2 + MSP1, Appomattox water 1&2 + MSP1, Buffalo soil 1&2 + MSP1, and Buffalo water 1&2 + MSP1 (Figure 5).

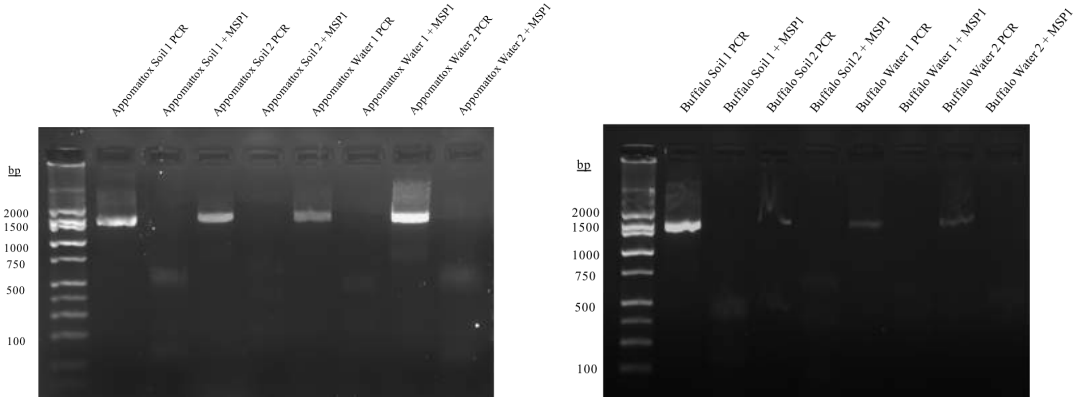


Figure 5. Gel electrophoresis results of each PCR product and sample + MSP1.

DNA Sequences

The DNA was sent to EurofinsGenomics and all eight colonies came back with successful DNA sequences. Each prokaryote was identified using BLAST alignment (Table 3). The selected colonies were compared to current research to further identify that they were the correct BLAST Match (Figure 6).

Location	Closest BLAST Match	Colony Description	Environment	% Similarity	Gaps
App River Soil	*Rugamonas rubra*	red-pigmented, circular shape	river water	99%	4/1017
App River Soil	*Bacillus toyonensis*	white, irregular shape	probiotics in animal nutrition	99%	1/968
App River Water	*Flavobacterium aquidurense*	dark orange, circular shape	hard water creek	99%	4/810
App River Water	*Pseudomonas fragi*	white, circular shape	freshwater	99%	1/873
Buff Creek Soil	*Flavobacterium saccharophilum*	light orange, irregular shape	soil and freshwater	98%	2/874
Buff Creek Soil	*Bacillus simplex*	white, circular shape	forest soil	100%	0/982
Buff Creek Water	*Pseudomonas tolaasii*	white, circular shape	white mushrooms	99%	2/783
Buff Creek Water	*Janthinobacterium agaricidamnosum*	purple, punctiform shape	cultivated mushrooms	99%	1/861

Table 3. Top alignment results from BLAST with the percent similarity, along with the number of gaps found in the sequence, and the environment it had been previously found in.

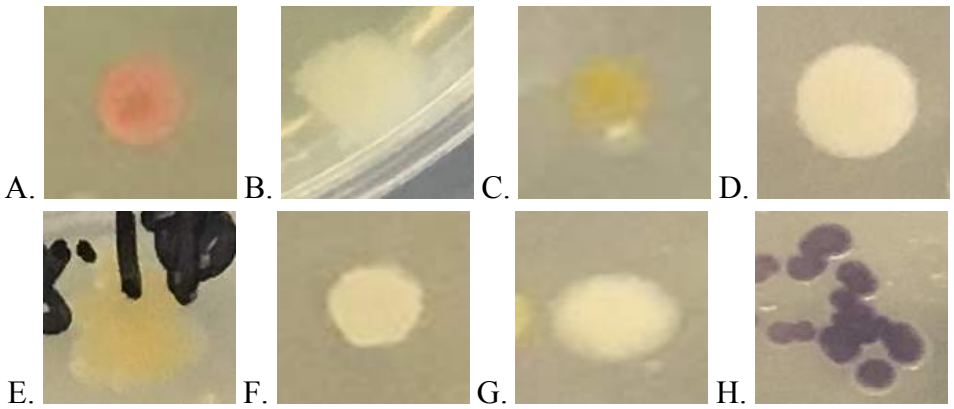


Figure 6. Selected colonies that were identified after DNA sequencing and BLAST alignment.
(A) *Rugamonas rubra* (B) *Bacillus toyonensis* (C) *Flavobacterium aquidurense*
(D) *Pseudomonas fragi* (E) *Flavobacterium saccharophilum* (F) *Bacillus simplex*
(G) *Pseudomonas tolaasii* (H) *Janthinobacterium agaricidamnosum*

Discussion

The goal of this experiment was to investigate how agricultural and recreational pollution effect the microbial diversity of water and sediment collected from Buffalo Creek and the Appomattox River. We hypothesized that the more pollution that is found in the samples, the less amount of microbial diversity will be present. We collected eight samples: two water and two soil from locations in Buffalo Creek and the Appomattox River. All eight of these samples were sequenced and were successfully identified using BLAST alignment.

The amount of water pollution was high in Buffalo Creek and low in the Appomattox River based on the pH, salinity, phosphate, and fluoride levels (Table 1). This correlated with the amount of microbial abundance in the water of each location: Buffalo Creek had a lower abundance and the Appomattox River had a higher abundance (Figure 2). However, the soil samples from each location were much higher in diversity and abundance and the sediment was not tested so we cannot conclude if there was a difference between the diversity of water and the diversity of soil. We can, however, conclude that microbial diversity is much higher in sediment than it is in water. This can be concluded because creek/river water is constantly moving and sediment just stays on the bottom of the river where diversity increases.

The conclusions made from our experiment are very comparable to other studies. The study of river water and pollution suggest that microbial diversity in sediment-water was low due to environmental factors, such as organic pollutants and ammonia, which is what was concluded in our experiment (Ibekwe A, Ma J, Murinda S). We also had the same results as another study

that concluded the microbial diversity of sediment samples was modified by the organic pollution, but not largely (Toes A, Finke N, Kuenen J, Muyzer G). We found that sediment was very diverse, so the pollution did not have very much effect on the microbial diversity. In a third study conducted in China, authors reported that pollution inhibits microbial diversity in soil (Chen J, He F, Zhang X, Sun X, Zheng J, Zheng J). It has been found that microbial diversity in soil is affected by pollution, specifically organochlorines (Miguel AS, et al). A study done on the effects of petroleum toxicity to microbial diversity also concluded the same results as our study (Srivastava J, Naraian R, Kalra SJS, Chandra H).

Most of the prokaryotes we identified were expected based on other published work; they had been previously found in the same type of locations we had identified them in. However, *Pseudomonas tolaasii* and *Janthinobacterium agaricidamnosum* were previously found in white mushrooms and cultivated mushrooms so we were unsure of why they were found in creek water (Abou-Zeid MA. & Lincoln, S.P., Fermor, T.R., and Tindall, B.J). *Rugamonas rubra* has been previously found in river water in England. It was reported as being red-pigmented, so we knew our BLAST identification was correct (Austin, D.A, and Moss, M.O.). *Bacillus toyonensis* has been found in Japan and is used as a probiotic in animal nutrition (Jimenez, G., et al). This species was not surprising because there is a farm very close to the collection site of this sample. *Flavobacterium aquidurense* has been found in Northern Germany in a hard water creek (Cousin, S., Pauker, O., and Stackebrandt, E.). Although found this species in a river, it was still classified as “hard” (Table 1). *Pseudomonas fragi* was previously found in China where it was used for treating urban sewage by detrification (Cristina Pellegrini et al). *Flavobacterium saccharophilum* has been found in a lowland river in silt and freshwater in the River Wey in Surrey, U.K. (Agbo J.A.C., and Moss, M.O.) *Bacillus simplex* has been found in Morocco in arid soil and is also known as *Bacillus maroccanus* (Delaporte, B., and Sasson, A.).

If this study were to be repeated we would take a closer look at the amount of pollution and types in the soil since this was not tested in this experiment. We would also test the levels of nitrogen in the soil and see if that had a correlation to the types of microbial diversity. The information concluded from this study adds evidence that pollution does negatively impact microbial diversity. This is important because it truly shows that pollution is hurting our environment even on this small of a scale. Microbial diversity is very important because it is at the bottom of the food chain. If microbial diversity is severely impacted, then a chain of negative

events will occur and this can end up hurting the biosphere. This study should be repeated on a larger scale and can be adapted to see if pollution will decrease the diversity in our oceans.

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