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Variations in Stream Sediment Microbial Communities Across a Natural Climate Gradient in Eastern Puerto Rico

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VARIATIONS IN STREAM SEDIMENT MICROBIAL COMMUNITIES ACROSS A NATURAL CLIMATE GRADIENT IN EASTERN PUERTO RICO

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Abstract:

Six months following the passage of Hurricane Maria across Puerto Rico, water and sediment samples were taken at eight locations along a precipitation gradient to assess the impact of sediment upwelling, organic matter input, and other heavy precipitation-associated influences on water chemistry and microbial populations. Eight sites were broken up into wet (291-440 cm precipitation/yr), dry (140-290 cm precipitation/yr) and reference streams (controls). The hypotheses were as follows: wet environments will be different from dry environments in terms of the number of colony forming units. Furthermore, it is hypothesized that wetter environments should support a greater number of CFUs and that microbial species will show preference for either wet or dry environments. Lastly, it is hypothesized that ion concentrations will be greater in wetter environments and will affect microbial growth. There was no significant relationship between the number of colony-forming units and sampling location (wet vs. dry, wet vs. reference, dry vs. reference), as determined by a Kruskal-Wallis test. No species showed a specific preference for one environment over the other. No pathogenic organisms were recovered through biochemical analyses, though opportunistic pathogens were present. 9 of the 14 organisms identified are a normal part of warm-blooded flora. Contrary to my hypothesis, and in support of Lindsey (2018), I found higher concentrations of dissolved ions at sites receiving less annual rainfall (Figures 5, 6, 7 and 8). Heavier precipitation results in dilution, while evaporation results in accumulation of ions (Lindsey 2018). This could affect microbial metabolism, as well as alter community structure as climate continues to change (Prayitno *et al.* 2018). This study provides a snapshot of microbial composition after precipitation events, which may broaden understanding of the affect heavy rain events have on microbes and, subsequently, human and ecosystem health.

Introduction:

Precipitation is water released from clouds in the form of rain, snow, sleet and hail. It is the process in the water cycle which returns water to the earth from the atmosphere, sustaining life. Climate influences all living things (Zhang *et al.* 2016). It dictates how and where an organism can survive. The amount of precipitation an area receives influences many elements of an ecosystem; for a stream, these include: run-off and discharge, water chemistry, habitat formation, and food sources (Bae 2019). Variations in rainfall affect all these characteristics, influencing the survival of organisms which live within them (Bae 2019, Zhang *et al.* 2016).

The amount of precipitation a stream receives alters its physical characteristics, such as the shape and depth of incision patterns (Han *et al.* 2014). Precipitation patterns impact the volume of water entering a stream, which changes velocity, and, thus rates of physical and chemical weathering (Han *et al.* 2014). Additionally, heavier precipitation events increase nutrient additions and can contribute to as much as 400% and 80% of average annual input of nitrogen and phosphorous, respectively (Rostami *et al.* 2017). Precipitation events may change as climate fluctuates, further affecting shape, depth, water chemistry, turbidity and many other aspects of a stream. (Runkle *et al.* 2018, Sullivan 2014).

Changes in the amount of rainfall an area receives will alter water chemistry and quality and, thus, the assemblage of organisms at any given location (Zhang *et al.* 2016). If changes in water chemistry alters the functionality of even one species in a community, it is likely that those effects will be seen throughout the entirety of the ecosystem (Smee 2010). Changes in ecosystem composition could change stream health, and subsequently human health.

This study focuses on the effect of changing precipitation patterns on microbial population composition in Puerto Rico. These data provide a glimpse of current microbial assemblages and introduce the question of how future climate scenarios will change microbial population dynamics and human health.

Puerto Rico, the eastern most island of the Greater Antilles, measures 140 km east to west and 40 km north to south at their maximums. The landscape is dominated by a central mountain range, the Cordillera Central. Further to the east are the mountainous areas associated with El Yunque National Forest and its surroundings. Upland areas account for about sixty percent of the land area. The climate is tropical marine with an average temperature of 26°C. Rainfall is generally evenly distributed throughout the year, but January through April is drier than the rest of the year. Total precipitation varies spatially due to mountains and trade winds. Rainfall ranges from a low of 90 cm in the south-central region to around 500 cm in the area around El Yunque. Due to the orientation and extent of the interior mountains, there are no large river systems in

Puerto Rico. Rather, there are numerous small streams that originate in the mountains and flow across the coastal lowlands to the ocean. The precipitation gradient described is large, and as such, it may affect water chemistry, sediment chemistry, and thus the microbes supported at each sampling location. In 2011, Puerto Rico received 2-4 times the amount of rainfall than average recordings (Runkle *et al.* 2018). This resulted in significant flooding across most of the island, apart from the southwest region (Runkle *et al.* 2018); which highlights the importance of this study.

In this study, microbial composition in small streams across Puerto Rico receiving varying amounts of precipitation annually were analyzed to assess differences in the number of colony forming units (CFUs) in wet (300-440 cm mean annual precipitation) vs. dry (140-299 cm MAP) and reference (control, i.e. minimal human interaction) streams. It is hypothesized that wet environments will promote a greater number of CFUs than dry environments, as precipitation causes suspension of sediments and nutrients (Martín-Díaz *et al.* 2017). Furthermore, all organisms require water to survive; hence wetter environments should support a greater number of CFUs. It is also hypothesized that ion concentrations will be greater in areas receiving more annual rainfall and will have an effect on microbial growth. Lastly, it is hypothesized that wet environments will support a greater number of colony forming units and a greater number of species than dry

environments for the same reason that total number of colony forming units will be greater in wet environments; moisture promotes bacterial growth.

Shift in climate will most likely result in precipitation changes due to warmer sea surface temperatures and higher moisture holding capacity of the atmosphere, especially in tropical regions such as Puerto Rico (Runkle *et al.* 2018). This study will provide a snapshot of current microbial populations at sites receiving various mean annual precipitation amounts with the purpose of further understanding how precipitation affects microbial assemblages, and to use these data to extrapolate future scenarios.

Materials and Methods:

Sampling

In this study, six streams were chosen to analyze. Streams were divided into two categories: wet (291-440 cm/yr) and dry (140-290 cm/yr). There were three streams assigned to each category based on how much rainfall the area surrounding the stream receives annually. Two reference streams, Rio Mameyes and Espiritu Santo, were chosen to compare results. They are reference streams because they are considered pristine and human interaction is minimal. The only other physical difference between the reference streams and study streams is the bedrock—reference streams are the extrusive equivalent of a pluton. Both reference streams receive approximately 300 centimeters of rain annually. These

reference streams were used to identify any differences attributable to anthropogenic sources in the six study streams. The objectives are to determine

Water and sediment samples were taken from the aforementioned eight streams in eastern Puerto Rico in March of 2018: Rio Mameyes, Esperitu Santos, Rio Blanco, Rio Humacao, Rio Yabucoa, Rio Maunabo, Rio Jacaboa and Rio Apeadero—each receiving successively less rainfall annually. USGS flow data was not validated for any of our sampling sites. Therefore, flow conditions may not be accurately calculated for the time of sampling. The procedure for sampling followed USGS National Field Manual for the Collection of Water-Quality Data (U.S. Geological Survey), and samples were taken at USGS gauging stations. Samples for all water analyses were taken in 1 L sterile amber bottles made from polypropylene. Bottles were acid-washed, then submerged in 70% isopropyl alcohol for 30 seconds, completing the disinfection process. Wet bottles/caps were placed face down on sterile gauze pads and left for 5 minutes to air dry. Bottles were then tightly capped and remained that way until sampling occurred. Sample bottles were pre-rinsed with stream water and submerged approximately 12 inches below the surface, facing upstream. Sample bottles were filled and tightly capped upon removal. Samples were placed in a cooler, on ice, until they could be frozen. Water samples remained frozen and were analyzed in January 2019. Sterile DI was used for field blanks and was plated following the same protocol as the other samples.

Sediment samples were taken in 100 mL plastic sampling containers. Bottles were sterilized following the same protocol as above. At each site, sampling containers were submerged face-down and cored into the river floor. Once the bottle was full, it was capped and placed in a cooler, on ice, until it could be refridgerated. Sterile DI was used for field blanks and was plated following the same protocol as the other samples.

Sediment Microbiology:

Several biochemical tests were performed to help identify cultured organisms. Results were plugged into an online database, which returned the species that each culture matched. The analyses performed are summarized below.

Preparation of glassware and media

All glassware was autoclaved at 15 psi and 121° C for 15 minutes to ensure proper sterilization. Broths (glucose, lactose, sucrose) were purchased as dehydrated media, then diluted and sterilized in house. Upon arrival to the lab 500 mL of DI water was measured into four flasks and five grams of each solid sugar (glucose, lactose, sucrose) were weighed and added to their individual flask to create 1% carbohydrate solutions. 10 grams of solid urease was added to the fourth 500 mL flask to create a 2% urease solution. After the broths were made, they went into the autoclave at the settings above. Solutions were poured into sterile, labeled test tubes (9 mL each) and received 1 mL of phenol red to identify

pH changes after inoculation. All tubes were tightly capped to prevent contamination. All media not made in the lab (eosin methylene blue agar, trypticase soy agar, MacConkey agar, and mannitol salt agar) was made and shipped to SUNY Brockport from Carolina Scientific or Fisher Scientific.

Initial sediment dilutions/plating on TSA

The lab bench was wiped with 70% isopropanol before and after each work day to maintain a clean work space. Gloves were worn throughout the inoculation process, as well as during cleanup, and were changed frequently to reduce the likelihood of contamination. Hands were washed with antimicrobial soap before gloves were put on and after they were removed.

Sediment samples were weighed and added to sterile glass test tubes, applying a ratio of 1-gram wet mass sediment to 9 mL sterile DI water. Sediment broths were gently agitated with a sterile glass rod for 2 minutes to provide proper aeration and nutrient suspension. Controls were run with the same protocol, except sterile DI (without any sediment additions) was used. Dilutions were left for 24 hours at 37°C to allow bacterial multiplication to occur. From each dilution, a 10^{-2} dilution was produced to avoid over-crowding of colonies when plated. 0.1 mL of each broth (10^{-2} dilution) was administered to a trypticase soy agar (TSA) plate labeled by location of sediment obtained. TSA plates were purchased from Carolina Scientific and were used as general growth media. Each broth was plated twice. All further analyses were also performed in duplicates to

ensure quality control. Inoculation of agar was accomplished via micropipette (instrument disinfected, tips sterilized) and sterile cell spreaders. After inoculation, lids were placed back on the dishes, turned upside down, and left at 37°C for 48 hours. For additional QC measures, Mameyes sediment was inoculated on 3 separate TSA plates. For control measures, one plate was left exposed to laboratory air for 15 minutes and one was left un-inoculated to assess potential contamination by air and/or pre-poured plates.

Colony differentiation based on physical attributes and gram stain technique

After 72 hours, individual plates were examined for the number of different colonies, as well as the physical features they possessed, including: size (mm), color, shape, edge, opacity, and texture. The number of colony forming units (CFUs), the number of colony types, and the number of colonies belonging to each type per plate was recorded. After the number of different colony types were identified, a gram stain was performed to characterize colonies into either gram-negative or gram-positive bacteria, as well as to clarify morphology.

Inoculation of selective/differential media from TSA plates: Mannitol Salt Agar (MAC) for detection of *Staphylococcus* species, MacConkey Agar (MAC) for Gram-negative, lactose-fermenting microorganisms, and Eosin Methylene Blue Agar (EMB) for differential and slightly selective isolation of Gram-negative enteric microorganisms (bacilli).

Individual, distinguished colonies were transferred from original TSA agar plates onto each of the three differential plates using an inoculation loop and following aseptic procedures. All plates (MSA, MAC, EMB) were sectioned into four equal parts to maximize the number of colonies that could be inoculated per plate and to reduce waste. Each section of the differential plates was labeled with features that distinguished a particular colony type from the other colonies, such as shape, color, or some other physical identifier. Colony forming units used for inoculation were isolated from the others, i.e. the borders of the colony used were not touching another colony of a different type. This was done to reduce the likelihood of cross-contamination by another species. All individual colony types were inoculated onto all media types, regardless of gram stain results. This served as additional gram stain indicators, i.e. MAC and EMB agar select for gram negative bacteria, thus gram-positive bacteria will not grow on these media. Plates were left for 48 hours at 37°C.

Glucose/Sucrose/Lactose

All three carbohydrate broths were made in the lab from dehydrated solute. A 1% solution of each sugar was made (process explained above in glassware prep). All solutions were autoclaved at 121˚C and 16.2 PSI for 15 minutes after prep to ensure sterilization. 5 mL of phenol red pH indicator was added to each of the 3 carbohydrate broths. 3 sets of 28 (duplicates) sterile test tubes were labeled by colony identifiers and set up on the lab bench next to a label

of either 'glucose', 'lactose' or 'sucrose'. A disinfected pipette and sterile tips were used to transfer broths from the beaker of solution into test tubes. Tips were changed between each transfer and the pipette was disinfected between different carbohydrate broth transfers. Each set of test tubes received 10 mL of one of the sugar broths. Tubes were immediately flamed and capped upon solution transfer. Test tubes were flamed before and after the inoculation of bacteria. Tubes were left for 48 hours at 37°C post-inoculation. Tubes were analyzed for any color changes at this time. A yellow tube indicated the ability of the inoculated organism to ferment that carbohydrate and change the color of the pH indicator.

Hemolysis

From the original TSA agar plates, isolated colonies were inoculated onto 5% sheep blood mixed with TSA to determine hemolysis patterns. Inoculated plates were left to incubate for 24 hours at 37°C. Plates were analyzed for the presence of growth and red blood cell lysis (symptomatic of hemolysin protein). Results were recorded as alpha (partial breakdown of red blood cells), beta (complete breakdown of red blood cells), gamma (no breakdown of red blood cells) or no growth.

Starch Hydrolysis

Starch plates were sectioned in half and labeled by colony identifiers. The original TSA plates were used for inoculation of starch plates. Inoculated plates

were left to incubate for 48 hours at 37°C. Plates were flooded with iodine and analyzed for a clear zone around the bacterial growth to indicate the presence or absence of the extra-cellular α-amylase enzyme.

Gelatin Hydrolysis

12% gelatin broth tubes were labeled by colony identifiers and inoculated using the stab technique. Caps and tops of tubes were flamed upon opening and before re-sealing the cap. Inoculated tubes were left for 48 hours at 37°C. Tubes were analyzed for the presence of growth and then placed in an ice bath for 30 minutes. Medium liquefaction after removal from the bath was symptomatic of the presence of gelatinase enzyme.

SIM

SIM agar was utilized for both sulfur production and tryptophanase biochemical tests, as well as for motility determination. SIM agar tubes were labeled by colony identifiers and inoculated using the stab-streak technique. Inoculated tubes were left for 24 hours at 37°C. Tubes were first analyzed for growth patterns determinative of motility, as well as production of ferric sulfide precipitate, which is produced when sulfur reduction occurs. Five drops of Kovac's reagent was then added to determine tryptophanase activity. Development of a cherry red layer on top of the media indicated a positive result for the presence of tryptophanase enzymes (hydrolyzation of tryptophan).

Simmon's Citrate Agar

Simmon's citrate agar slants were used to determine citrate utilization. Test tubes were labeled by colony identifiers and inoculated using the streak technique. Citrate tubes were left for 48 hours and analyzed for color change. Citrate slants that went from green to blue indicated alkaline carbonate and bicarbonate production, increasing pH, and changing the media color.

Catalase

Hydrogen peroxide was used to determine the presence of catalase enzymes, which break down toxic free radicals into water and oxygen molecules. An inoculating needle was used to place a small amount of bacteria onto a sterile card. Hydrogen peroxide was administered to the bacterial mass using a dropper. If bubbles were present, the colony was determined to be catalase-positive.

Urease

Urea broth was made in the lab. 20 grams of dehydrated urea were added to 980 mL of sterile DI water to create a 2% urea solution. 5 mL of phenol red was added to the solution as a pH indicator. 20 sterile test tubes were labeled by colony identifiers and placed in test tube holders. A disinfected pipette and sterile pipette tips were used to transfer 10 mL of the urea broth into each of the test tubes. The tops of the test tubes were flamed and capped immediately following the addition of solution. Test tubes were also flamed before and after the

inoculation of bacteria. Tubes were left for 48 hours at 37°C post-inoculation. Tubes were analyzed for any color changes at this time. A pink tube indicated the ability of the organism to ferment urea (urease enzyme) and change the color of the pH indicator.

Coagulase

Only gram-positive cocci were tested for coagulase presence, as it is a significant identifier for pathogenic staph strains. A BBL™ Staphyloslide™ Latex kit was used to detect the presence of protein A, indicative of *Staphylococcus aureus.* Distinct colonies were transferred from the TSA plate onto test cards. A solution containing blue polystyrene latex particles sensitized with fibrogen and IgG (a type of antibody) was administered to the bacteria. A second portion of the same bacteria was administered a control latex solution that contained desensitized latex, meaning no agglutination would occur, regardless of species.

Water Chemistry:

Total Phosphorus

Total phosphorus was analyzed using the ChemWell®-T discrete sample analyzer. We followed EPA 365.1 standard operating procedure. Samples were digested with ammonium persulfate and sulfuric acid to convert all phosphorus to orthophosphate (Verdouw *et al.* 1978). Ammonium molybdate and antimony

potassium tartrate react with orthophosphate to create a complex which is reduced upon the introduction of ascorbic acid. If there is phosphorus present, the water will become blue, correlating with the concentration of phosphorus in the water. In other words, the darker the color the higher the concentration of phosphorus is. The analyzer performed all of this for us and, also, provided a standard curve for concentration determination.

Sediment Moisture

Sediment samples were weighed into crucibles, each approximately 1 gram each. For QC measures, 3 replicates of a randomly chosen sample, J, were made. Weight of the crucible alone was recorded first, followed by weight of the crucible plus the addition of the sediment sample. Desiccated sediment and crucible were weighed to obtain the final value used for % water content calculation.

Ion Chromatography

Water samples were brought to ALS Environmental in Rochester for calcium, magnesium, potassium and chloride analysis upon return to NY. ALS Environmental uses Thermo Scientific Dionex Ion Chromatography (IC) ICS-2100 systems. The Thermo Scientific Dionex™ ICS-2100 Ion Chromatography System (Dionex ICS-2100) performs ion analyses using suppressed or non-suppressed conductivity detection (Thermo Fisher Scientific Inc 2012).

Data Analysis

Statistics and figures were analyzed in Excel. Water chemistry data from 1972 to 2016 (with the exception of the year 1989, in which Hurricane Hugo occurred) was collected for each sampling site from USGS public record and used in a box plot. USGS samples were collected following The National Field Manual for the Collection of Water-Quality Data (USGS 2009). Ion chromatograph data was overlain the existing box plot and compared to the 44-year medial value. Phosphorus data were analyzed via a regression analysis, with the x variable being precipitation in centimeters and the y being phosphorous in parts per million. From this analysis an r-value was provided—allowing determination of the correlation between annual rainfall and phosphorous concentration.

Biochemical tests were arranged in an excel file, then plugged into ABIS software to obtain species results (Stoica and Sorescu 2012). Bergey's Manual of Determinative Bacteriology was used to confirm results (Bergey and Holt 2000). The independent variable for all tests was the amount of annual rainfall. The dependent variable varied depending on which hypothesis was being tested. I analyzed by total CFUs in each of the three environments (wet vs. dry vs. reference), by number of species supported at each site (wet vs. dry vs. reference) to assess diversity. I also analyzed by the number of CFUs per species at each environment (wet vs. dry vs. reference) to identify any microbial preference for one environment over the other.

Microbiology data were analyzed using a Kruskal-Wallis test, as these data violate the normality assumption, determined by a histogram (figures 3 and 4). For the first hypothesis, total number of all colonies (regardless of species) at what we considered dry environments/treatments (sites RB, H, Y), wet environments/treatments (MB, J, A) and reference streams/controls (ES, M) were run in one Kruskall-Wallis test. This conveys if any of the treatments (dry, wet, reference) has significantly more or less colony forming units. It encompasses all species. For the second hypothesis, I looked at the number of CFUs from each individual species in each treatment (to determine preference for a treatment), so another Kruskall-Wallis test was done based on treatment and the number of CFUs, but only for one species at a time—totaling 14 different tests (number of species I found). Diversity was done the same way, but the number of different species in each treatment was used as a dependent variable as opposed to colony forming units.

Results:

Sediment Microbiology

Approximately 64% of all the species identified exist as normal human flora (Table 2). None of the species identified pose a risk to human health, unless an individual or individuals are immunocompromised (i.e. children, the elderly, HIV/AIDS patients, and other immunosuppressed individuals). There was not a significant difference in the total number of colony forming units for wet, dry or

reference streams as determined by a Kruskall-Wallis test ($p = >0.05$) (Table 3). None of the species we identified showed a preference for any treatment, i.e. wet, dry, or reference sites ($p = >0.05$) (Table 4). Diversity was the same at all sites, also determined by a Kruskall-Wallis test ($p = >0.05$) (Table 5).

Ion Chromatography

Contrary to my hypothesis, calcium, magnesium, sodium and chloride have positive trends as the stream gradient progresses from most annual rainfall to least (Figures 5, 6, 7 and 8). Measured values for each of the four parameters generally existed above the USGS median for each location, though this was not the case for all data points.

Total Phosphorus

USEPA (1986) has a recommended limit of 0.05 mg/L for total phosphates in streams that enter lakes and 0.1 mg/L for total phosphorus in flowing waters. Total phosphorous exceeded 0.05 mg/L at all sites and was exceedingly high at Rio de Apeadero—1.4 mg/L (Figure 9). There was not a strong correlation ($r= 0.35$) between annual rainfall and phosphorous concentration--though, as noted, Rio de Apeadero had much higher levels. This is possibly due to being downstream from a city and adjacent to agricultural fields.

Discussion:

In this study, bacterial species existing along a precipitation gradient in eastern Puerto Rico were identified and enumerated with the purpose of gaining a better understanding of how bacterial populations are represented at various mean annual precipitation sites, and also to use these data to extrapolate how climate change might affect these bacterial populations. In a similar study, Froeschke (2015) utilized a natural precipitation gradient in southern Africa to assess nematode infestation of Rhabdomys pumilio, the striped mouse. Results showed a significant positive correlation between infestation rates and mean annual rainfall. Both studies are of importance, as they may help predict climate related threats to ecosystem health (Froeschke et al. 2015).

Increasing human population has led to an increase in fossil fuel and resource exhaustion, amplifying greenhouse gas emissions and increasing the temperature and moisture holding capacity of the atmosphere. This means that rain events may be more intense in the future, and this effect will most likely be amplified in tropical regions (Emanuel *et al.* 2005, Herren et al. 2016, Shade et al. 2012). It is expected that the precipitation gradient across the island of Puerto Rico will shift, and annual rainfall will increase across all sites (Runkle *et al.* 2018).

We know that bacteria are our biggest recyclers—they govern the process of environmental decomposition and re-assimilation of usable nutrients back into

soils and sediments for the utilization of other organisms (Forehead *et al.* 2013). They contribute significantly to the cycling of carbon and other nutrients, such as nitrogen. Density and diversity of species are major contributing factors to these processes and may be affected by large storm events and drought (Forehead *et al.* 2013). Puerto Rican climate is predominately tropical rainforest—meaning the effect of climate change on precipitation will be more drastic here than in other regions (Runkle et al. 2018). Changes in microbial composition and metabolism from changing precipitation patterns may affect decomposition rates (Sydney et al. 2018), which may result in significant changes in carbon cycling. The consequences of this are still unknown.

It is common knowledge that not all bacteria from an environmental sample such as sediment are able to be cultured, meaning it is very likely that not all species that existed within the sediment samples were accounted for. In the future, DNA amplification should be utilized to ensure that most species are included in the analysis. The bacterial data gathered for this study is preliminary and will be used as background knowledge for studies to come. Out of all 14 species identified, 9 can be attributed to humans and/or other warm-blooded organisms (Table 1A, 1B, 1C). The species considered to be originating from environmental (sediment, water) sources, and were found in reference streams, are: *Pseudomonas aeruginosa, Bacillus nealsonii, Bacillus subtilis, Bacillus atrophaeus,* and *Paenibacillus barcinonensi* (Earl *et al.* 2008). Finding several

Bacillus species in reference streams as well as in experimental streams is to be expected. *Bacillus* species are among the most prevalent and resistant of bacteria—they produce spores which can survive through extreme conditions such as desiccation, chemical treatment, and UV radiation (Nicholson *et al.* 2000).

Higher concentrations of human-related microbes in streams receiving more annual rainfall were expected, because increased precipitation leads to more run off, which increases the chances of contact with humans and animals, as opposed to sites receiving less precipitation and/or less run off (Tornevi *et al.* 2014). With projected increase of extreme weather events, notably precipitation in Puerto Rico, water quality will decrease, and presence of pathogenic bacteria (originating from humans and livestock) is expected to increase—also why higher concentrations of human and animal-associated bacteria in areas where annual precipitation is greater is expected (Tornevi *et al.* 2014).

The force exuded by more intense precipitation will most likely put some microbial species under stress due to physical and chemical changes of habitat (Shade *et al.* 2012). Genetic variation within microbial populations may decrease from the intensity of these changes—resulting in significant kill-offs and affecting a variety of gene expressions, overall population functionality and survivability (Herren *et al.* 2016, Shade *et al.* 2012). Genetic diversity is crucial, as it allows populations to remain variable and express or possess alleles that may help some

individuals of a population survive disturbances (Shade *et al.* 2012). If these events continue to escalate in intensity, a single storm event, or a series of events, may cause a major reduction in genetic diversity across the entirety of the study gradient—making populations more susceptible to extinction (Shade *et al.* 2012).

Additionally, flooding from storm events often contains overflow from waste water treatment plants as well as other industrial practices, and provides a suitable environment for bacterial proliferation and gene transfer (Yu *et al.* 2018). Flood waters post-Hurricane Harvey had elevated concentrations of *Escherichia coli*, a fecal coliform, in comparison to historical levels (Yu *et al.* 2018). More alarming, antibiotic resistance genes originating from anthropogenic sources were also elevated in comparison to prior recorded levels (Yu *et al.* 2018). Other studies report similar findings. Pruden *et al.* 2012 described that the antibiotic resistant gene *sul*1 (sulfonamide) was present in significantly higher numbers downstream of concentrated animal feeding operations as well as waste water treatment plants, both anthropogenic sources. Precipitation events increase the transport of these genes, and increase the likelihood of gene transfer, and, thus, a greater presence of resistant bacteria (Pruden *at al.* 2012, Yu *et al.* 2018). Most of the study sites in this study are downstream from cities, making gene transfer more likely. This is critical because it shows that waterways, especially after heavy precipitation events, are important transport mechanisms of bacteria and antibacterial resistance genes, which is a growing human health concern (Pruden

et al. 2012, Yu *et al.* 2018). In 2011, Puerto Rico received 2-4 times the amount of rainfall than average recordings (Runkle *et al.* 2018). This resulted in significant flooding across most of the island (Runkle *et al.* 2018). This amount of precipitation increases chances of water-borne infection in the population, especially in poverty-stricken areas where health care is limited, and disease is more prevalent (Hunter and Arbona 1995). Although the species recovered in this study were opportunistic and not necessarily pathogenic, the likelihood of them causing disease is amplified by weak health and immunity (Hunter and Arbona 1995). The likelihood of opportunistic pathogens picking up resistance genes during wet conditions is also greater, increasing the risk of disease (Pruden *et al.* 2012). Additionally, un-culturable bacteria were not accounted for in this study, but the presence of fecal indicators such as *Enterobacteriaceae* and *Escherichia* across all study sites indicates potential for fecal contamination of local water supply. Because most of the sampling sites in this study are downstream from populated areas and agricultural fields, the likely-hood of bacterial contamination of infrastructure will increase greatly as precipitation events intensify.

Besides human health concerns, there should also be a concern for the effect that changing precipitation patterns will have on the surrounding ecosystem. We know that some dissolved ions alter the pH of water; we also know that some ions such as sodium and chloride form salts (Lindsey 2018). Based on past patterns, it is predicted that changes in climate will result in

fluctuations of ion concentrations in waterways—especially in small streams such as the ones in this study (Lindsey 2018, Ramírez *et al.* 2014, Baker-Austin *et al.* 2017). Heavier precipitation will result in dilution of ions, while evaporation will result in accumulation (Lindsey 2018). Contrary to the hypothesis in this study, and in support of Lindsey (2018), higher concentrations of dissolved ions at sites receiving less annual rainfall were found (Figures 5, 6, 7 and 8). Both accumulation and depletion of ions could affect microbial metabolism, as well as change community structure (Prayitno *et al.* 2018).

For example, Prayitno (1995) investigated the prevalence of brine shrimp larvae (*Artemia nauplii*) diseased with a bacterial isolate similar to *Vibrio harveyi*and and *Photobacterium phosphoreum* in Indonesia. The occurrence of the outbreaks was correlated with the rainy season, which resulted in dilute, brackish coastal conditions. Exposure of these pathogens to low-salinity conditions (10 and 15 ppt) resulted in significantly increased mortality of *Artemia nauplii* when compared to normal ocean salt concentration treatments (27 ppt) (Prayitno *et al.* 1995). This shows that environmental conditions, such as ion concentration, may affect the prevalence of disease not only in humans, but also in other organisms, affecting food chain dynamics (Prayitno *et al.* 1995). To assess the effect of other ion concentrations on bacterial species, an experiment could be designed in which microbial species in the study area are cultured and subject to varying concentrations of a given ion, while all other variables are controlled.

All of the streams sampled in this study were small, low flow streams. Projected future climate scenarios will most likely alter their shape and depth due to projected shifts in precipitation (Sullivan 2014). Physical and chemical weathering will change ion concentrations in water, amplifying what we are seeing in this study—lower concentrations at sites receiving less precipitation. The effects of this fluctuation on microbes and other organisms is unknown and makes a great future study question.

Conclusion

The bacterial findings of this study were not expected. There was no relationship between treatment and microbial numbers or diversity from these data. In the future, instead of culturing microbes, gene amplification will be used. Gene amplification accounts for all species in a sample, regardless of if they are culturable or not. Additionally, more streams will be used for sampling to increase the power of statistical testing.

Ion concentrations were higher at sights receiving less rainfall, which could play a roll in microbial population functionality. In order to test this, cultured microbial species from each of the study sites should be subject to various concentrations of analyte (magnesium, calcium, sodium, chloride). The number of colonies should be recorded at each concentration to assess how additions affect microbial populations. This is an important next step, as ion concentrations showed a clear correlation with annual precipitation.

With the threat of increased storm intensity caused by surface sea water warming and increased moisture holding capacity of the atmosphere (Emmanuel 2005), precautions should be taken for both human life and the environment. Water acts as a transport system for many pathogens that may pose a health risk upon exposure or consumption, and hurricanes and other heavy rain events amplify the rate of that transportation (Martín-Díaz *et al.* 2017, Herren *et al.* 2016). With projected increases in precipitation across the gradient described, more cases of disease could occur as a result, and not just in humans.

Understanding how precipitation affects microbial mechanisms may increase knowledge of large storm events and the affects they have. This will allow scientists and policy makers to make better planning and implementation decisions, as well as create factual, accurate resiliency and environmental impact assessments.

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Table 1A: Table one shows the biochemical analyses that were utilized in this study. From these results, I used Advanced Bacterial Identification Software and Bergey's Manual of Determinative Bacteriology to obtain species result

Species	Bacterial colony color/description	Size (mm)	Gram stain/morphology	MSA	EMB	MAC
Enterobacter aerogenes (78%)*	Mucoid, white, opaque	6	Gram-negative bacilli	no growth	good growth, pink	good growth, pink
Escherichia hermanni (84.6%)	Mucoid,Opaque white, circular, entire	1.75	Gram-negative bacilli	no growth	good growth, pink	good growth, pink
Pseudomonas aeruginosa (98.39%)	Transparent beige, mucoid, circular, entire, umbonate	3.5	Gram-negative coccobacilli	no growth	good growth, colorless	good growth, colorless
*Citrobacter sedlakii (81.2%)**	Opaque beige, circular	3.75	Gram-negative bacilli	no growth	good growth, blue	good growth,dark pink
Bacillus nealsonii (92.8%)	Pink-brown, irregular, undulate, transparent	4.5	Gram-positive bacilli	good growth, colorless	no growth	no growth
*Serratia marcescens (80.72%)**	Pink, opaque, round, entire	0.3	Gram-negative bacilli	no growth	good growth, pink	good growth, pink
Streptococcus caballi (99%)	Dark yellow, transparent, irregular, undulate	0.3	Gram-positive cocci	no growth	good growth, colorless	good growth, colorless
Paenibacillus barcinonensis (98.93%)	White, circular, curled, opaque	15	Gram-positive bacilli	no growth	good growth, dark pink	good growth, pink
*Micrococcus luteus (78.03%)**	Yellow-orange, small colonies, circular, entire	0.35	Gram-positive cocci	good growth, colorless	no growth	no growth
*Staphylococcus lugdunensis (83.6%)**	Cream-yellow, small colonies, round, entire, opaque	0.1	Gram-positive cocci	good growth, yellow	no growth	no growth
Bacillus subtilis (78.2%)	Transparent yellow, irregular	12.5	Gram-positive bacilli	good growth, yellow	no growth	no growth
*Edwardsiella tarda biogroup 1 (83.9%)**	Light yellow, irregular, transparent	10	Gram-negative bacilli	no growth	good growth, blue	good growth, dark pink
Bacillus atrophaeus (88.23%)	Cream white, opaque, inorganic, undulate, vertical growth	13	Gram-positive bacilli	no growth	no growth	no growth
*Proteus mirabilis (95%)**	White, circular, curled, opaque	6.13	Gram-negative bacilli	no growth	good growth, blue	good growth, dark pink
*Alcaligenes faecalis (90.12%)**	Brown, opaque, irregular, small colonies	0.27	Gram-negative bacilli	no growth	good growth, colorless	good growth, colorless

Table 1B: Table one shows the biochemical analyses that were utilized in this study. From these results, I used Advanced Bacterial Identification Software and Bergey's Manual of Determinative Bacteriology to obtain species results.

Species	Hemolysis	Starch	Gelatin	Indole	HS2	Citrate
Enterobacter aerogenes (78%)*	poor growth	positive	negative, good growth	negative	negative	positive
Escherichia hermanni (84.6%)	beta	negative	positive, good growth	positive	negative	negative
Pseudomonas aeruginosa (98.39%)	no growth	negative	positive, good growth	negative	negative	positive
*Citrobacter sedlakii (81.2%)**	gamma	no growth	negative, good growth	positive	negative	positive
Bacillus nealsonii (92.8%)	gamma	negative	negative, no growth	negative	negative	negative
*Serratia marcescens (80.72%)**	gamma	negative	positive, good growth	negative	negative	positive
Streptococcus caballi (99%)	alpha	positive	negative, good growth	negative	negative	positive
Paenibacillus barcinonensis (98.93%)	alpha	negative	positive, good growth	positive	negative	positive
*Micrococcus luteus (78.03%)**	gamma	positive	positive, good growth	negative	negative	negative
*Staphylococcus lugdunensis (83.6%)**	beta	negative	positive, good growth	negative	negative	negative
Bacillus subtilis (78.2%)	beta	negative	positive, good growth	negative	negative	negative
*Edwardsiella tarda biogroup 1 (83.9%)**	gamma	positive	negative, good growth	positive	negative	negative
Bacillus atrophaeus (88.23%)	beta	negative	negative, good growth	negative	negative	negative
*Proteus mirabilis (95%)**	alpha	positive	negative, good growth	positive	negative	negative
*Alcaligenes faecalis (90.12%)**	gamma	negative	positive, good growth	negative	negative	negative

Table 1C: Table one shows the biochemical analyses that were utilized in this study. From these results, I used Advanced Bacterial Identification Software and Bergey's Manual of Determinative Bacteriology to obtain species results.

Species	Catalase	Urease	Coagulase	nitrate	motility
Enterobacter aerogenes (78%)*	negative	negative	NA	negative	positive
Escherichia hermanni (84.6%)	negative	negative	NA	positive	positive
Pseudomonas aeruginosa (98.39%)	positive	negative	NA	positive	positive
*Citrobacter sedlakii (81.2%)**	negative	positive	NA	negative	positive
Bacillus nealsonii (92.8%)	positive	negative	NA	negative	positive
*Serratia marcescens (80.72%)**	negative	negative	NA	positive	positive
Streptococcus caballi (99%)	positive	negative	negative	negative	positive
Paenibacillus barcinonensis (98.93%)	negative	negative	NA	negative	positive
*Micrococcus luteus (78.03%)**	positive	positive	negative	negative	negative
*Staphylococcus lugdunensis (83.6%)**	negative	negative	positive	negative	negative
Bacillus subtilis (78.2%)	negative	negative	NA	negative	negative
*Edwardsiella tarda biogroup 1 (83.9%)**	negative	negative	NA	negative	positive
Bacillus atrophaeus (88.23%)	negative	negative	NA	negative	positive
*Proteus mirabilis (95%)**	negative	positive	NA	positive	positive
*Alcaligenes faecalis (90.12%)**	negative	negative	NA	negative	positive

Table 1D: Table one shows the biochemical analyses that were utilized in this study. From these results, I used Advanced Bacterial Identification Software and Bergey's Manual of Determinative Bacteriology to obtain species results.

Species	Glucose	Lactose	Sucrose
Enterobacter aerogenes (78%)*	+	+	-
Escherichia hermanni (84.6%)	+	+	-
Pseudomonas aeruginosa (98.39%)	+	-	+
*Citrobacter sedlakii (81.2%)**	+	+	-
Bacillus nealsonii (92.8%)	+	+	+
*Serratia marcescens (80.72%)**	+	-	+
Streptococcus caballi (99%)	+	-	+
Paenibacillus barcinonensis (98.93%)	+	+	-
*Micrococcus luteus (78.03%)**	+	+	+
*Staphylococcus lugdunensis (83.6%)**	+	+	+
Bacillus subtilis (78.2%)	+	+	-
*Edwardsiella tarda biogroup 1 (83.9%)**	+	-	+
Bacillus atrophaeus (88.23%)	+	-	+
*Proteus mirabilis (95%)**	+	-	-
*Alcaligenes faecalis (90.12%)**	+	+	+

Table 2: Total colony counts of each species for each location sampled. Values were obtained by taking the mean of each colony type on duplicate plates. Sites are organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams.

	Reference	Reference	Wet	Wet	Wet	Dry	Dry	Dry
	ES	M	RB	H	Y	MB	J	A
*Enterobacter aerogenes (78%) *ϕ*	3	5	1	2	10	4	2	1
*Escherichia hermanni (84.6%) *ϕ*	0	1	0	0	6	4	2	0
Pseudomonas aeruginosa (98.39%) ϕ	7	3	12	4	16	5	14	2
*Citrobacter sedlakii (81.2%) **	2	8	8	4	0	1	3	18
Bacillus nealsonii (92.8%)	0	0	0	0	1	0	11	1
*Serratia marcescens (80.72%) *ϕ*	0	0	3	0	2	0	0	0
Streptococcus caballi (99%)	1	0	0	0	5	0	4	2
Paenibacillus barcinonensis (98.93%)	0	0	0	0	2	0	0	0
*Micrococcus luteus (78.03%) **	2	0	5	0	0	0	5	2
*Staphylococcus lugdunensis (83.6%) *ϕ*	1	0	5	0	0	0	1	3
Bacillus subtilis (78.2%)	1	1	0	0	1	0	0	6
Edwardsiella tarda biogroup 1 (83.9%)	2	4	1	0	1	1	0	0
Bacillus atrophaeus (88.23%)	0	0	5	6	1	0	0	0
*Proteus mirabilis (95%) *ϕ*	0	3	1	0	3	1	0	4
Alcaligenes faecalis (90.12%) ϕ	3	2	1	0	0	1	2	4
TOTAL CFU PER mL	2200	2700	4200	1600	4800	1700	4400	4300
# SPECIES/SITE (RICHNESS)	9	8	10	4	11	7	9	10

= found in human GI tract or as part of normal flora

ϕ= opportunistic pathogen

Table 3: Kruskall-Wallis test results for total CFU (reference vs. wet vs. dry)

Test Stat (H)	Critical Value	df	Significance
0.745	5.791	2	NOT SIGNIFICANT

Table 4: Kruskal-Wallis test results for species preference (reference vs. wet vs. dry). * = found in human GI tract or as part of normal flora, ϕ= opportunistic pathogen.

	Test Stat (H)	Critical Value	df	Significance
*Enterobacter aerogenes (78%) *ϕ*	3.073	5.791	2	NOT SIGNIFICANT
*Escherichia hermanni (84.6%) *ϕ*	1.143	5.791	2	NOT SIGNIFICANT
Pseudomonas aeruginosa (98.39%) ϕ	2.291	5.791	2	NOT SIGNIFICANT
*Citrobacter sedlakii (81.2%) **	0.67	5.791	2	NOT SIGNIFICANT
Bacillus nealsonii (92.8%)	2.291	5.791	2	NOT SIGNIFICANT
*Serratia marcescens (80.72%) *ϕ*	2.545	5.791	2	NOT SIGNIFICANT
Streptococcus caballi (99%)	0.7	5.791	2	NOT SIGNIFICANT
Paenibacillus barcinonensis (98.93%)	0.636	5.791	2	NOT SIGNIFICANT
*Micrococcus luteus (78.03%) **	1.018	5.791	2	NOT SIGNIFICANT
*Staphylococcus lugdunensis (83.6%) *ϕ*	0.891	5.791	2	NOT SIGNIFICANT
Bacillus subtilis (78.2%)	2.143	5.791	2	NOT SIGNIFICANT
Edwardsiella tarda biogroup 1 (83.9%)	5.234	5.791	2	NOT SIGNIFICANT
Bacillus atrophaeus (88.23%)	5.727	5.791	2	NOT SIGNIFICANT
*Proteus mirabilis (95%) *ϕ*	0.143	5.791	2	NOT SIGNIFICANT
Alcaligenes faecalis (90.12%) ϕ	3.925	5.791	2	NOT SIGNIFICANT

Table 5: Kruskal-Wallis test results for diversity (number of species supported at each site (reference vs. wet vs. dry)

Reference	Reference	Wet	Wet	Wet	Dry	Dry	Dry
ES	M	RB	H	Y	MB	J	A
9	8	10	4	11	7	9	10

Test Stat	H	Significance
0.424	5.36	NOT SIGNIFICANT

Table 6: Table of ion concentrations organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams.

	Cl^- (mg/L)	Ca^{2+} (mg/L)	Mg^{2+} (mg/L)	Na^{2+} (mg/L)
ES	10.9	9.4	5.3	9.6
M	8	9.2	2.3	7.5
MB	24.1	20.2	8.3	22
Y	13.3	12.7	4.7	16.4
H	19.6	18.8	6.2	23.7
J	32.5	20.6	12.9	29.3
A	40.4	22	10.1	39.3

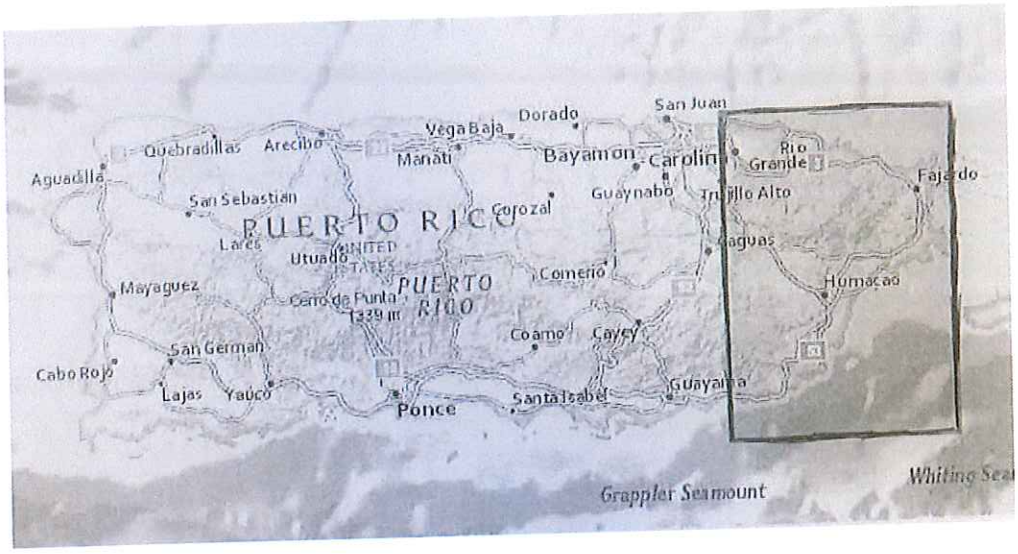

Figure 1A: The island of Puerto Rico with a subset showing the area our sampling sites existed within.

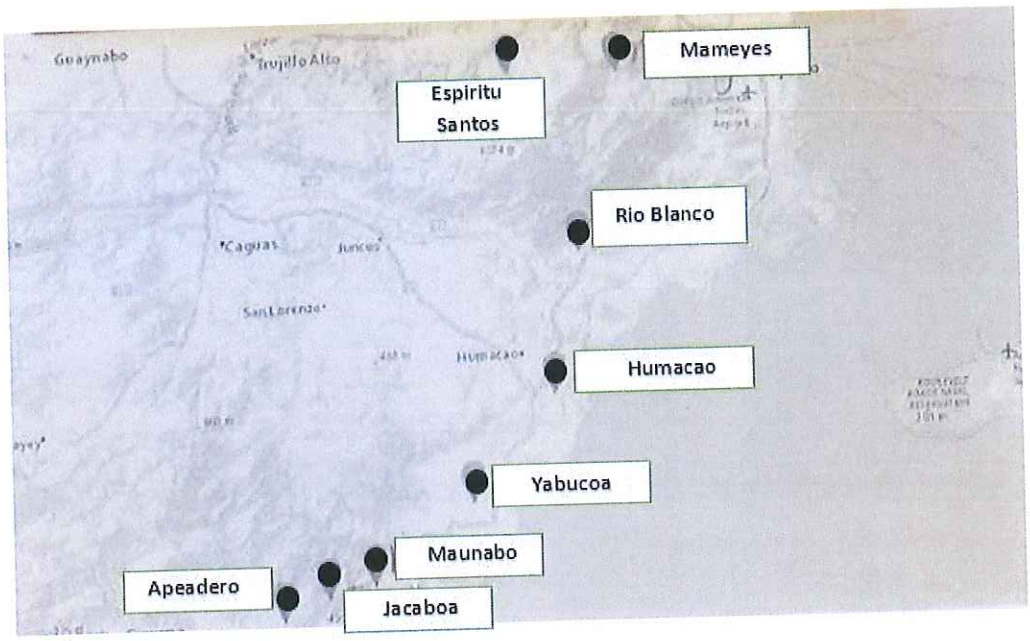

Figure 1B: Close up view of the subset from figure 1A—shows sampling locations along the eastern border.

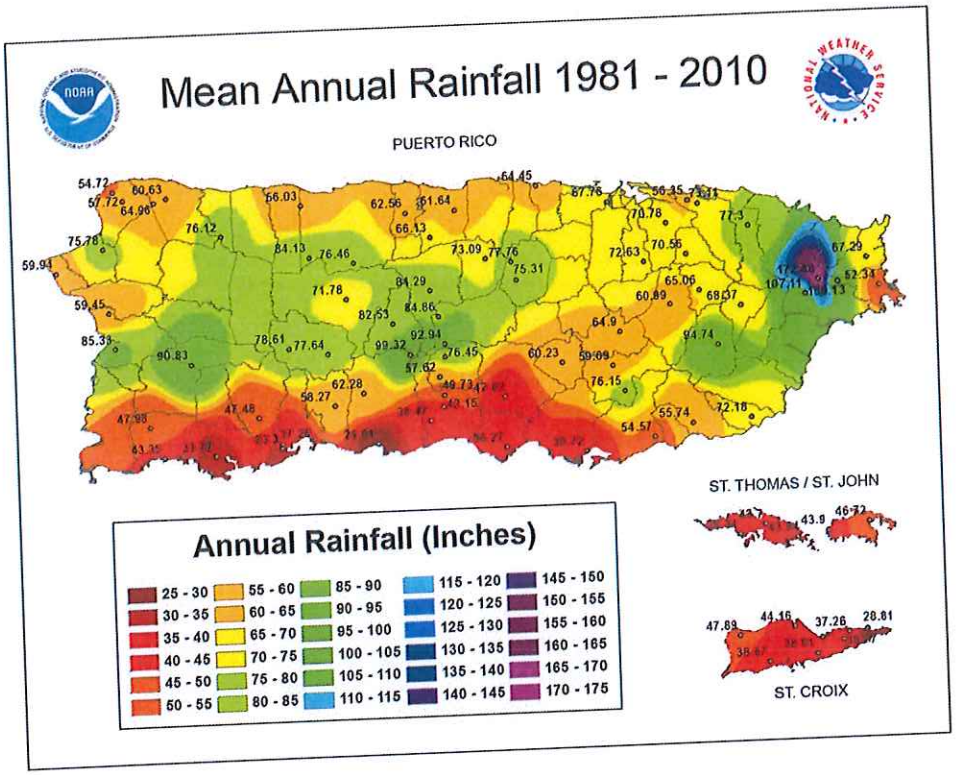

Figure 2: Map of annual rainfall across Puerto Rico—property of NOAA.

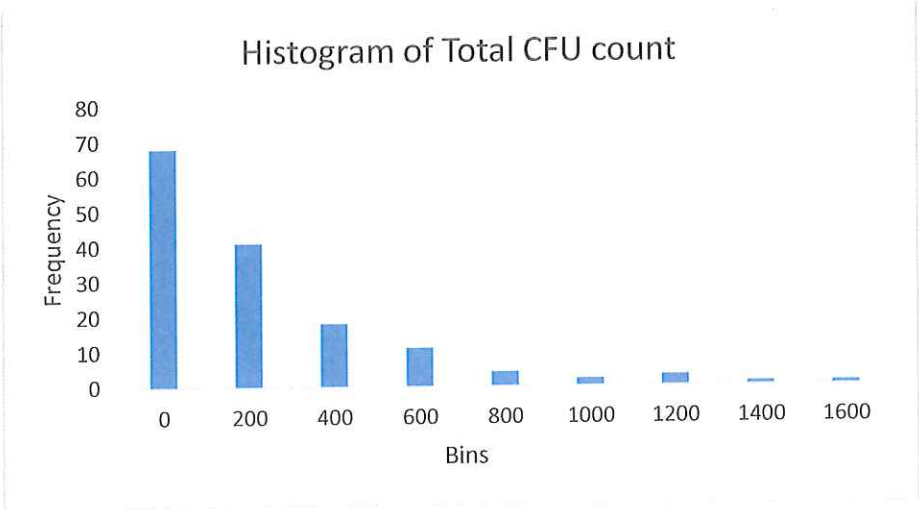

Figure 3: Histogram of total CFU data. Data is skewed-right.

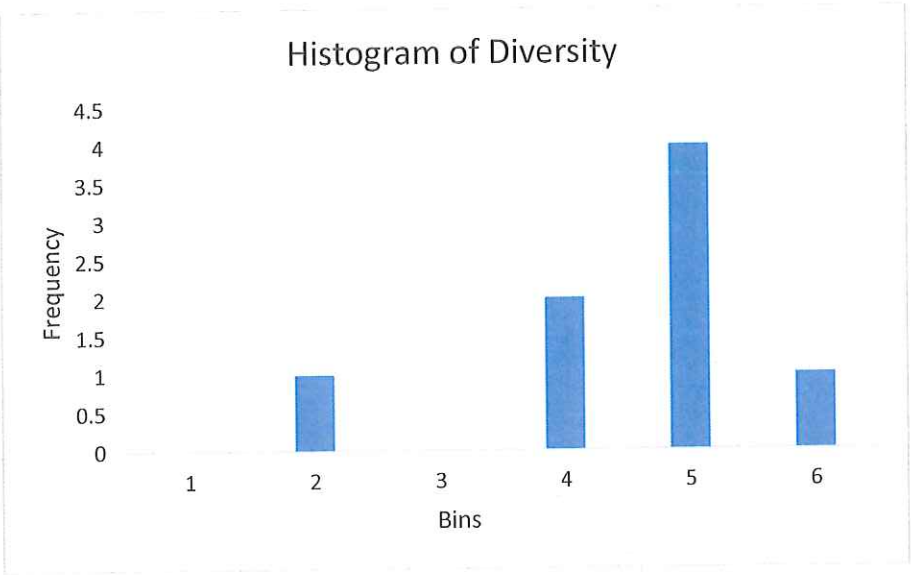

Figure 4: Histogram of diversity data. Data is skewed left.

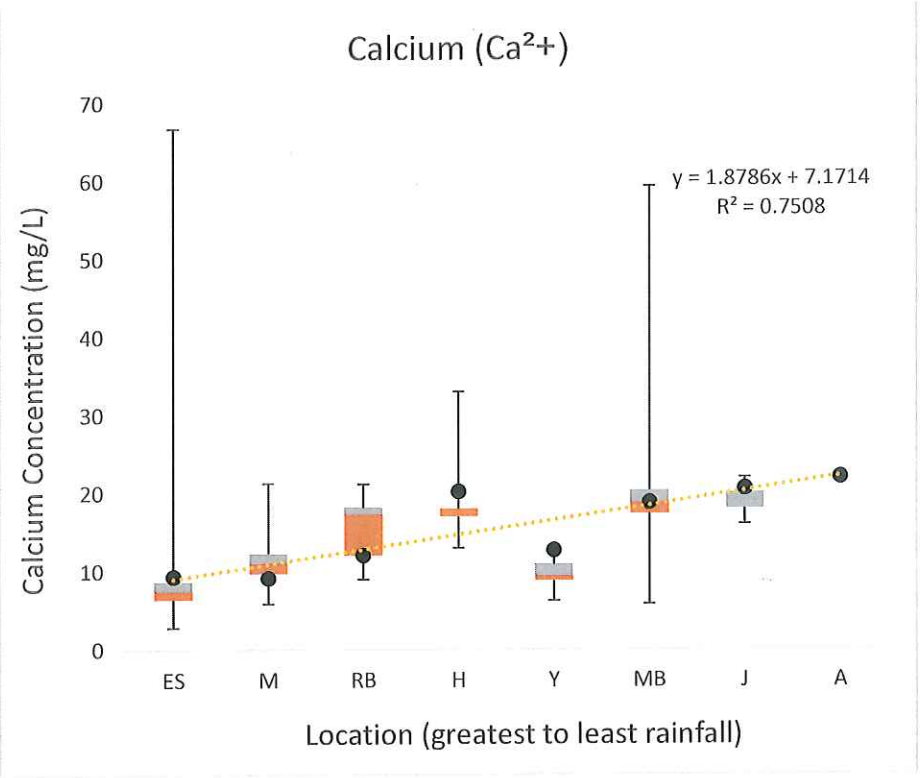

Figure 5: Box plot of calcium concentrations organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams (Esperitu Santos, Mameyes, Rio Blanco, Yabucoa, Maunabo, Jabacoa, and Apeadero). Plotted points represent post-hurricane measurements (6 months). The data source from the box plots is USGS archived data from the years 1972-2015. Río de Apeadero did not have USGS water chemistry data to compare results to.

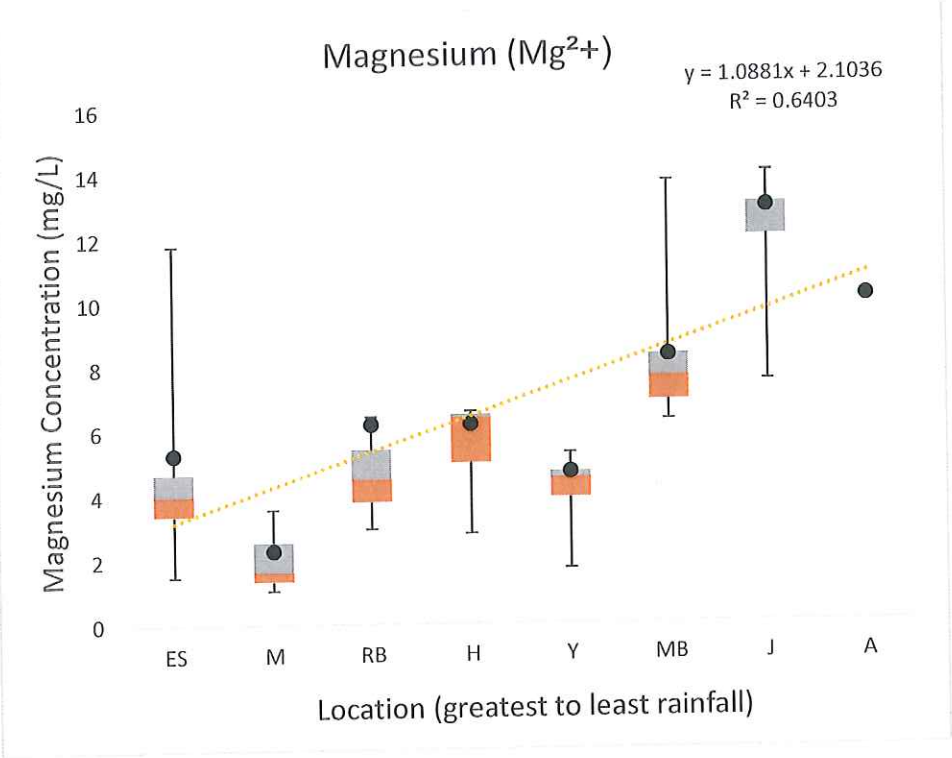

Figure 6: Box plot of magnesium concentrations organized by the site receiving the greatest amount of rainfall (Rio Blanco) to the site receiving the least amount of rainfall (Rio Apeadero), with the exception of the first two sites, which are reference streams (Esperitu Santos, Mameyes, Rio Blanco, Yabucoa, Maunabo, Jabacoa, and Apeadero). X variable is the amount of rainfall, and the Y variable is concentration of magnesium. Plotted points represent post-hurricane measurements (6 months). Río de Apeadero did not have USGS water chemistry data to compare results to.

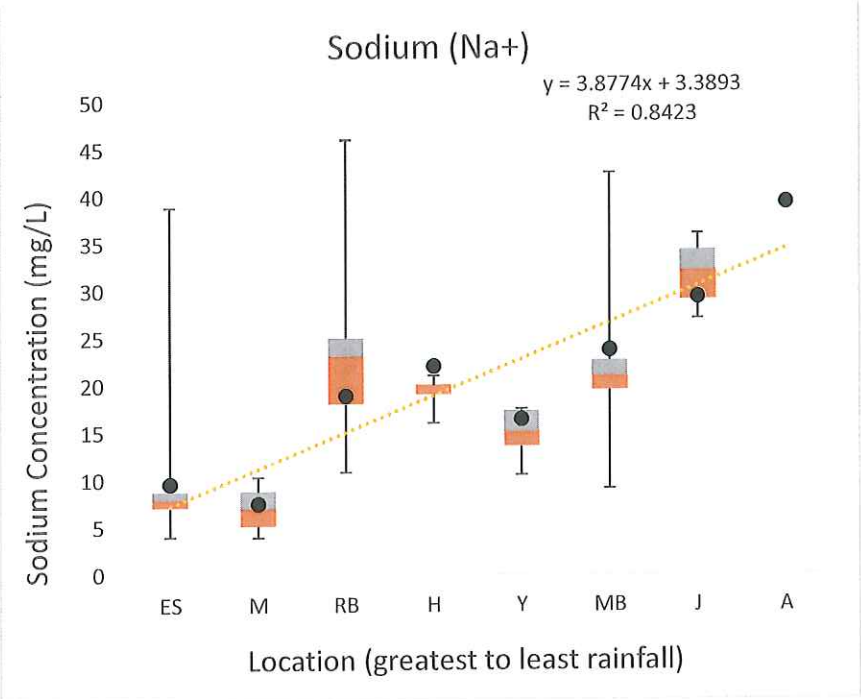

Figure 7: Box plot of sodium concentrations organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams (Esperitu Santos, Mameyes, Rio Blanco, Yabucoa, Maunabo, Jabacoa, and Apeadero). Plotted points represent post-hurricane measurements (6 months). Río de Apeadero did not have USGS water chemistry data to compare results to.

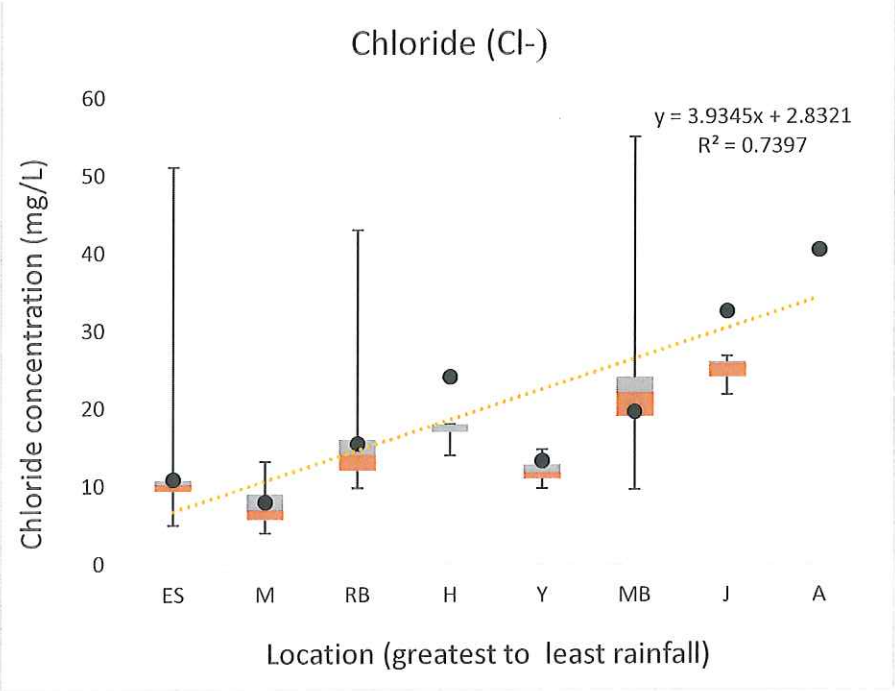

Figure 8: Box plot of chloride concentrations organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams (Esperitu Santos, Mameyes, Rio Blanco, Yabucoa, Maunabo, Jabacoa, and Apeadero). Plotted points represent post-hurricane measurements (6 months). Río de Apeadero did not have USGS water chemistry data to compare results to.

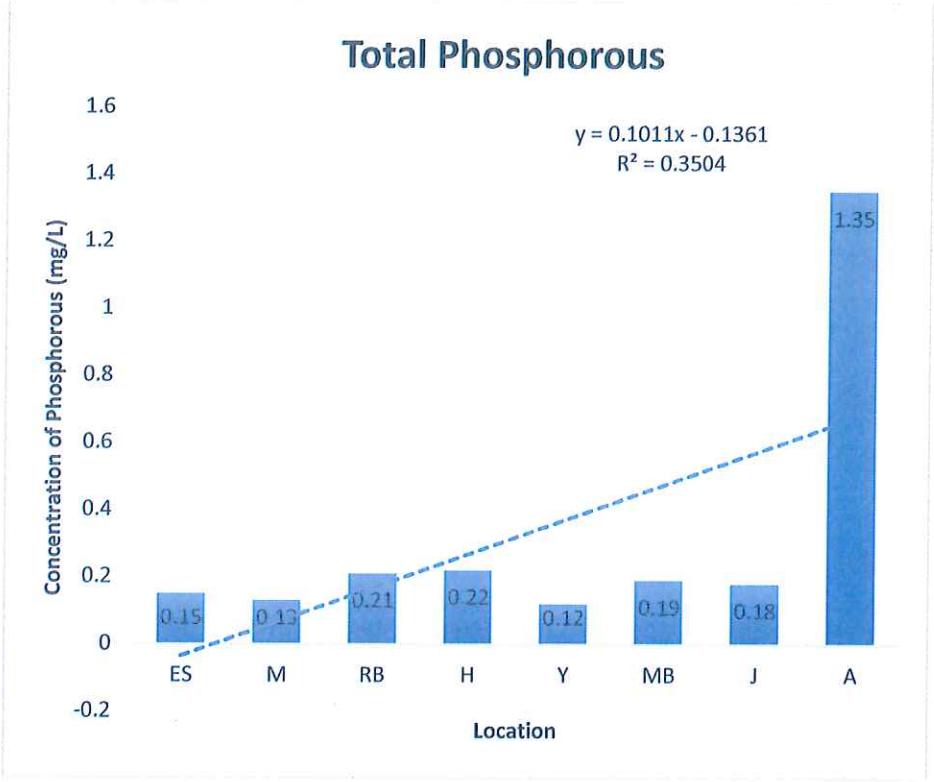

Figure 9: Bar graph showing measured total phosphorous (in parts per million) at each sampling location; organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams (Esperitu Santos, Mameyes, Rio Blanco, Yabucoa, Maunabo, Jabacoa, and Apeadero).

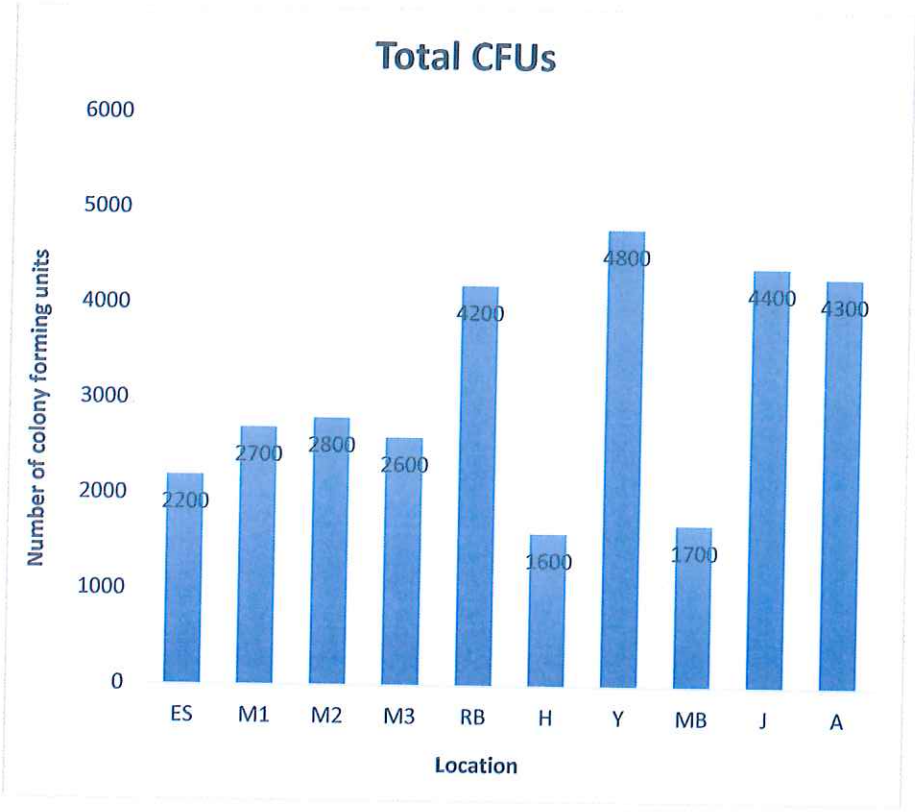

Figure 10: Bar graph showing the number of colony forming units per gram of sediment at each sampling location; organized by the site receiving the greatest amount of rainfall to the site receiving the least amount of rainfall, with the exception of the first two sites, which are reference streams (Esperitu Santos, Mameyes, Rio Blanco, Yabucoa, Maunabo, Jabacoa, and Apeadero