Environmental Bacteriophage Detection on Coastal Carolina University Campus

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Bacteriophages are viruses that infect bacteria. These viruses are found ubiquitously in the environment and are more abundant than any living organism on Earth, including bacteria. Eleven sites are designated for weekly sample collection on the campus of Coastal Carolina University. Water samples are filtered and amplified using strains of *E. coli B* and *E. coli K12* to allow potential coliphages in the sample to proliferate to detectable levels. Plaque assays are used as a microbial screen for the presence of bacteriophage. Samples that test positively using the microbial test are analyzed through a molecular test using PCR to identify the viral families and identify the coliphage. The results of this study illustrate the presence of bacteriophage on Coastal Carolina's campus and the identification of at least one of the desired viral families.The purpose of this study was to utilize bacteriophage as an environmental indicator of the presence of harmful bacteria in waterways on the campus of Coastal Carolina University and to identify bacteriophage that could be used to control bacterial blooms.

Introduction

Bacteriophages, also called phages, are viruses that parasitize bacteria for the purpose of reproduction. Lytic phages use bacterial cells as hosts to foster the rapid replication of phage genetic material, eventually causing lysis in the cell membrane and releasing new phages [1]. It is estimated that there are approximately 10^{31} phages on the planet, making them more common than every living organism combined, including bacteria [2]. One defining feature of bacteriophages is their specificity in suitable bacterial hosts, as most phages are only able to infect a subset of strains of a single bacterial species [3]. This phenomenon follows a general trend of evolutionary parasitism in the natural world in which a pathogen's adaptation to the specific features of a host result in a diminished ability to infect other organisms, resulting in the hyper-specialization that is attributed to most bacteriophages [4].

Bacteriophages' capability to significantly reduce the population of various harmful bacteria in an environment has been utilized with various degrees of success since their initial discovery by Frederick Twort in 1915 and Felix d'Herelle in 1917. Phage therapy became increasingly popular during the early 20th century after d'Herelle's first successful attempt to use bacteriophages to treat dysentery in 1919 [5]. d'Herelle, among other advocates for phage therapy, began producing phages to treat various bacterial infections, with commercial phage laboratories appearing in various established nations around the world, including Germany, Italy, the United States, Japan, and France [6]. However, with the introduction of antibiotics, phage therapy declined in popularity, allowing antibacterial measures to become the standard for treating bacterial infections [5].

Since their initial discovery in the early 20th century, antibiotics have been used to treat bacterial infections for decades; humanity's overuse of antibiotics, however, has allowed for the development of resistant bacteria that have resulted in thousands of deaths each year worldwide [7]. Specifically, the rates of antibacterial resistance in *Escherichia coli* have skyrocketed to levels of concern, reaching rates of roughly 60% in Asia, 30% in Latin America and southern Europe, and 5-10% in northern Europe and North America [8]. Without effective antibacterial restraints, pathogenic *E. coli* can pose a serious health concern, due in part to its ability to effectively spread via multiple mediums, including person-to-person contact, animal contact, and drinking water [9]. Moreover, a high concentration of *E. coli* in public waterways can indicate a hazardous environment in addition to water contamination [10]. The presence of coliphages, which are phages that parasitize coliform bacteria, such as *E. coli*, can indicate a moderate concentration of *E. coli* in an environment due to the phages' host specificity. Additionally, the discovery of coliphages in a contaminated region would provide a possible alternative to antibiotics when treating *E. coli* related infections.

In recent years, coliphages' potential in combating bacterial infections has gained attention, with coliphages being successfully used to treat UTIs and prevent food contamination [11]. Though essential to the success of coliphage treatment, the precise and selective nature of phages has served as one of the primary roadblocks when implementing phage therapy into common medical practice. Because most bacteriophages can only infect a single strain of bacteria, the effectiveness of coliphage therapy is limited when treating different *E. coli* infections, each of which would require unique, specific coliphage viruses [12]. Although a combination of phage and antibiotic treatment has been used in the past to treat coliform infections, the efficiency of this style of treatment can vary depending on the coliform's antibiotic resistance [13]. As such, the identification of coliphages in nature could assist in treating coliform infections by classifying specific viruses that can be used to safely destroy various harmful strains of *E. coli*.

This study is trying to locate and identify bacteriophages that lyse *E. coli* in any of Coastal Carolina University's eleven aquatic testing sites. Because of phages' aforementioned specificity for suitable bacterial hosts, the existence of these phages in Coastal Carolina's waterways would indicate an observable presence of the phages' respective host strains of *E. coli* on Coastal Carolina's campus. Additionally, identification of such bacteriophages could exhibit potential in combating bacterial blooms through natural processes. Because of their ability to swiftly eliminate large swaths of certain strains of bacteria without causing substantial harm to the surrounding ecosystem, bacteriophages have long been considered for use in repressing environmental bacterial blooms [14]. Identification of bacteriophages dependent on *E. coli* in Coastal Carolina's aquatic testing sites could be used in later research to aid in preventing the proliferation of strains *E. coli* in the natural environment through means of phage intervention. Additionally, by attempting to identify the detected coliphages' viral families, this study can effectively catalog natural phages that have potential uses in combating bacterial infections.

Traditionally, the collection of water quality data, weather condition reports, and catchment data determined the health of an aquatic environment [15]. However, no meaningful conclusions could be made using this traditional method as missing or insufficient data skews results. The alternative method to assess the health of an aquatic environment using bacteriophage detection proves to be a promising opportunity due to the unique specificity of phages' required host cells. By comparing the pH and temperatures of various waterways on Coastal Carolina's campus with the presence (or lack thereof) of coliphage, this study can also uncover environmental trends that may contribute to an increase or decrease in coliphage proliferation in Coastal Carolina's water systems.

Methods and Materials

Study Design

Eleven sample sites on Coastal Carolina's main campus were chosen, as depicted in figure 1. The experimental sample sites included both treated and untreated water bodies. The methods followed consisted of weekly collection, plaque assay tests, DNA extraction, PCR testing, and gel electrophoresis. 15-50 mL water samples were collected weekly from each of the eleven testing sites. Additionally, the pH and water temperature of each site were recorded to establish the relative environmental conditions during each collection.

Sterile Sample Filtration and Viral Amplification

The water samples were filtered with 10 mL Luer-Lok syringes outfitted with a 0.45 micron filter to remove unwanted material of microbes. Culture tubes with 4 mL of 2x LB broth were inoculated with 500 µL of either *E. coli B* or *E. coli K12* and 200 µL of the respective filtered environmental sample. This process of sample amplification allowed for any bacteriophage present within each sample to reproduce to the point of detection.

Plaque Assays

First, a plaque assay test was conducted to detect the presence of lytic bacteriophage. 2x LB agar plates served as a growth medium for the 2 different strains of *E. coli*. These plates were divided into quadrants following the bacterial inoculation. Three quadrats received the amplified site-specific sample while the remaining received the control, the bacterial culture. Any present phage lysed the growing bacteria and left a zone of lysis that has no bacterial growth. This zone of clearing serves as an indication that lytic bacteriophage are present in the amplified environmental sample.

DNA Extraction

Samples positive for lytic bacteriophage undergo DNA extraction to prepare for a molecular test. 100 µL of the amplified environmental samples was added to a microfuge tube. Using a centrifuge, the sample was spun down at the highest possible RPM for 5 minutes to separate the supernatant. Approximately 95 µL of suitable supernatant was added to a microcentrifuge tube with 5 µL of proteinase K. The samples were put in a shaking incubator for 60 minutes at 38ºC and then were placed on a heat block for 5 minutes at 95ºC to deactivate the proteinase K and to encourage further lysing.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

During the PCR, target viral genetic material was amplified using the following program

	Temp	Time
Initial denaturation	95°C	4min
Denaturation	94°C	30sec
Annealing	55°C	1min
Extension	72°C	2min

The particular test sought to determine the presence of two bacteriophage genes, GP23 and GP43. GP23 is a major capsid protein, and the primer design creates a fragment of 403 base pairs. GP43 is the core DNA polymerase of T4 replisome, and the primer design creates a fragment of 198 base pairs [16]. In each PCR tube, 25 µL of GoTaq Hot start master mix, 4 µL of either CPA, CPB, or CPO primer, and 21 µL of template DNA were added. The amplified samples were run on a 2% agarose gel at 90 volts for 1 hour, and a 100 base pair DNA ladder served as the reference.

Results

Routine physicochemical measurements illustrated that the pH and water temperature were within suitable ranges and were consistent throughout the study. The pH across the sample sites hovered around neutral to slightly alkaline while the temperature fluctuated between 50-75 °F. There was no statically significant difference between the average pH or average temperature (°F) across the experimental sample sites (p-value > 0.05). Since the physicochemical data demonstrated consistency and normalcy, they had no noticeable effect on bacteriophage presence or abundance (Figure 2).

Figure 1: Satellite Map of CCU's Main Campus with labeled sample site locations in white numbers.

As reflected in Figure 3, there were 28 confirmed microbial positives over the course of collection. Most microbial positives were observed in sample sites 9, 10, and 11 (See Figure 1 for locations), which comprise an untreated storm water drainage ditch that runs through the center of main campus. Aside from that observation, there were microbial positives seen at sample sites 1, 2, 3, 5, and 7 (See Figure 1 for locations). Microbial positives seen at these sample sites are significant since these sites have not tested positive for bacteriophage previously in microbial tests. However, bacteriophage presence has been detected using microbially testing at sample sites 9, 10, and 11 historically.

During microbial testing, a similar plaque morphology was observed as seen in Figures 4 and 5. On both plates, the lytic activity appeared to be similar, which allowed the connection to be drawn that the phage identified in each instance was the same or very similar, indicating an environmental reaction to a bacterial bloom of *E. coli*. As mentioned previously, these sample sites are connected, so that they experience comparable environmental stimuli.

Throughout the molecular testing, the O primer set, described in Table 1, repeatedly registered positive as reflected in figures 6, 7, and 8.

Discussion

The purpose of this study was to utilize bacteriophage as an environmental indicator of the presence of harmful bacteria in waterways on Coastal Carolina's campus and to identify bacteriophage that could be used to control bacterial blooms. Bacteriophage was successfully found and identified in many of the eleven sample sites. Of the detected bacteriophage, the O primer viral family reoccurred most frequently.

The most interesting results can be interpreted from the morphology and PCR of bacteriophage at collection sites 9, 10, and 11. The plaque assays displayed a strikingly similar morphology between detected bacteriophage in sites 9, 10, and 11. The PCR subsequently showed comparable results of consistent PCR positives for the CPO primer set for sample sites 9, 10, and 11 over time. This suggests that these observed viruses are either the same or very similar, and because of this, they infect similar strains of *E. coli*. For this trend to have been observed throughout the data collection, there must be a significant source of this bacteria existing in proximity to the sample sites which indicates a potentially negative environmental change.

In past years of data collection, there have been very few PCR positive results, which further indicates a recent environmental change. The pH and temperature data have consistent averages and have

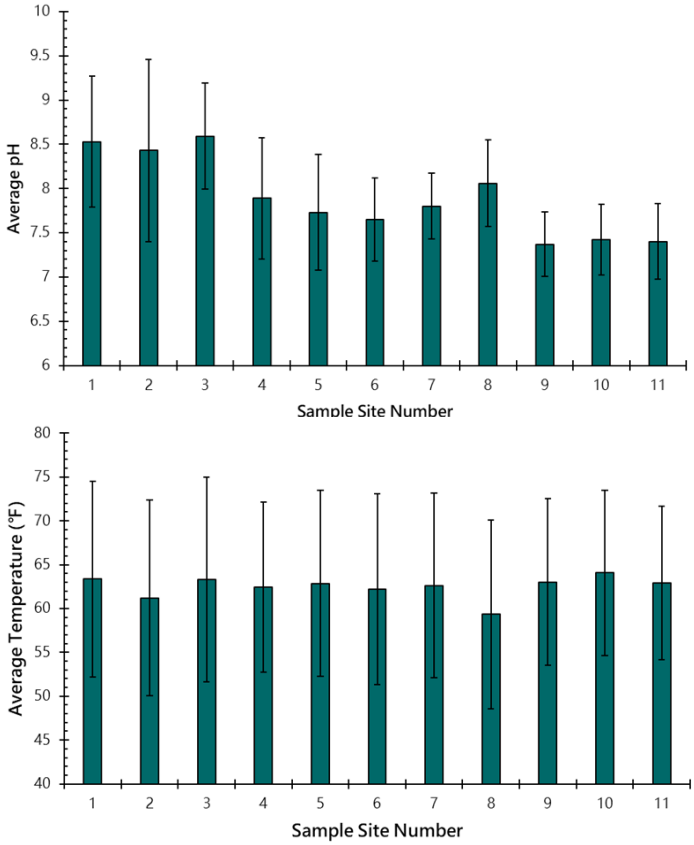

Figure 2: Physicochemical measurements from weekly water sampling. Error bars represent standard deviation. The coliphages were prominently detected in sample sites 9, 10, and 11, which are located along an untreated drainage ditch that runs through the main campus. These sample sites are all connected, so they experience

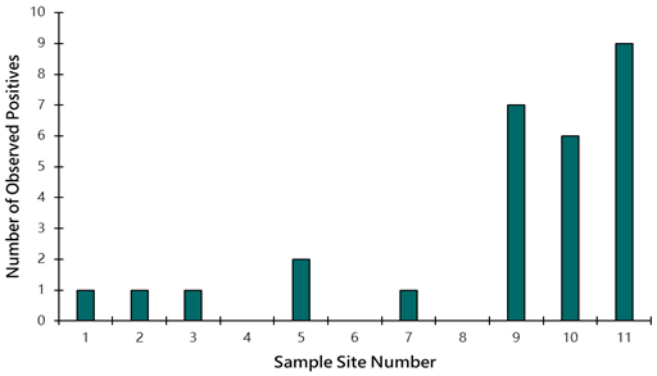

Figure 3: A graph depicting the number of observed bacteriophage positives across the eleven sample sites. Of the positives observed, the most notable results were similar morphologies on plaque assays from amplified environmental samples collected on the same day from related sample sites. Also, the visually comparable results shown on the gel were interesting as they represent samples taken on three different collection days.

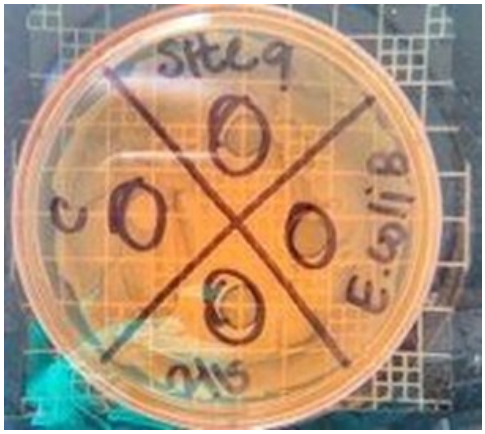

Figure 4: This photo is from site#9 on 2/15; exemplifies common morphology for plaque lysis caused by coliphage.

Figure 5: This photo is from site#10 on 2/15; exemplifies common morphology for plaque lysis caused by coliphages.

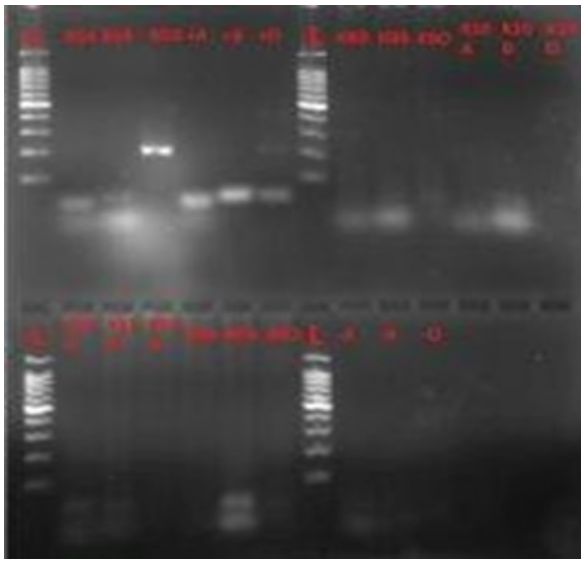

Figure 6: A positive molecular PCR result using the O Primer Set. In this agarose gel the O primer set shows the presence of Orf 43 in the fourth well. Samples in this gel were collected on 10/26 .

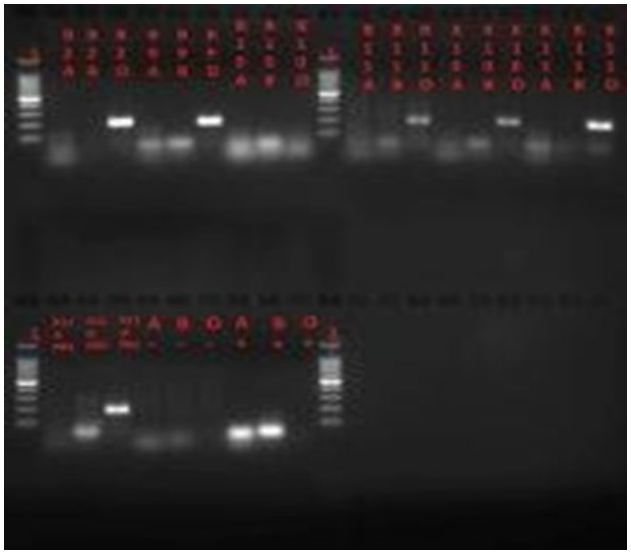

Figure 7: A positive molecular PCR result using the O Primer Set. In this agarose gel the O primer set shows the presence of Orf 43 in the a multitude of sample collections. Samples in this gel were collected on 2/15.

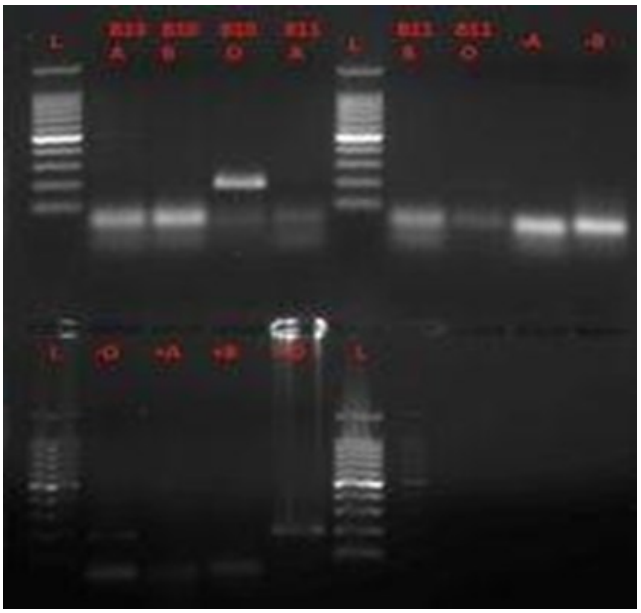

Figure 8: A positive molecular PCR result using the O Primer Set. In this agarose gel the O primer set shows the presence of Orf 43 in the fourth well. Samples in this gel were collected on 3/15.

remained consistent throughout previous data collection, indicating that these factors are not heavily influencing these blooms. Furthermore, these null environmental parameters coupled with the relatively novel proliferation of positive PCR results suggest the existence of an unknown factor, such as an coliphage infection within local wildlife, that could be in the fecal matter of infected hosts . This source must be experiencing longevity, which is allowing the methodology of using phage as an environmental indicator to be altered. This supports the hypothesis that phage can be used as a rapid and relatively inexpensive test to evaluate the health of an environment and to identify chronic environmental issues or contaminates, as the phage detection used in this study successfully identified a significant change in the region's environmental water quality.

Potential explanations of this environmental change could be due to changes in water treatment protocols. By using our method of phage detection, we can assess that the previous water treatment protocols were more effective in maintaining a low population of *E. coli.*

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Notes and References

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