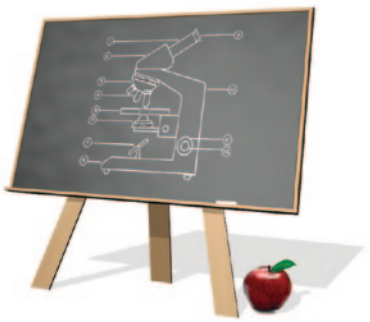
Introducing Students to Research: Electron Microscopy of Bacteriophages



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Note: The following describes the results of an educational project meant to teach laboratory microscopy and microbiology techniques and to illustrate the diversity and abundance of bacteriophages in different water sources. This project was undertaken by a high school student participating in The Scripps Research Institute's High School Student Research Education Program. The student was an intern at the National Resource for Automated Molecular Microscopy over the summer of 2009.

Introduction

Bacteriophages, as the name "bacteria-eater" suggests, are viruses that infect bacteria. Bacteriophages, often abbreviated as "phages," have receptors that bind to specific bacterial species, thus there are many types of bacteriophages. Once a phage interacts with its target bacterium, the phage injects its genetic material into the bacterial host where the phage is replicated to produce many new phages that then leave the host via cell lysis.

Although viruses are parasites that depend on a host for survival and aren't classified as truly living organisms, they have a large impact on our world. Phages are known to be the most plentiful biological entities on the planet, with one milliliter of seawater containing approximately 10^7 phages [1]. Phages that infect cyanobacteria, which fix carbon dioxide, have a major effect on the ocean's carbon cycle [2]. In addition, phages play a role in increasing the genetic diversity of bacteria, both through natural selection and by transferring genes across bacterial species [3].

Phages have the potential to be an important medical tool [4]. During World War II, countries that didn't have access to penicillin applied phages to wounds to prevent or treat infection [5]. Although small molecule antibiotics are more convenient and active against a broad range of bacterial pathogens, a growing resistance to modern antibiotics has become a major medical problem in recent years. Phages evolve much faster than their hosts, allowing them to adapt to changes in bacteria and making them a potentially more reliable antibiotic agent, once the correct phage is identified for the specific bacteria responsible for an infection. Bacteriophages have even been approved by the FDA as food additives to decrease the risk of food-borne illness.

Transmission electron microscopy (TEM) has played a major role in our understanding of bacteriophages. TEM allows viruses to be visualized and their structures to be analyzed on a near-molecular level. Here we describe a high school student's use of TEM to analyze various water samples. Because viruses are somewhat familiar to high school students, using TEM to observe viruses directly is naturally of interest to budding scientists. Phages and other microbial life were imaged, and one phage strain was propagated in the laboratory. The methods described are relatively straightforward and can be attained by a scientifically inclined high school student with the proper guidance and access to equipment.

Materials and Methods

Sample Collection. The marine samples were collected from Mission Bay Beach in San Diego; the sewage was from the South Bay Water Reclamation Plant, San Diego, CA, in the Tijuana River Valley; and the reclaimed water was from the irrigation system at The Scripps Research Institute, La Jolla, CA. A sample of approximately 500 mL of seawater was collected from Mission Beach. Sewage samples of 300 mL were collected after the tertiary process and at the influent stage from the water reclamation plant. To remove impurities and larger entities, 250 mL of the water from Mission Beach was filtered through a 0.45-µm filter using a 60-mL syringe into a new test tube. The sample of 300 mL of reclaimed water from The Scripps Research Institute was not filtered. All samples were stored at 4°C.

Grid Preparation. Copper grids (400-nm mesh), coated with a thin film of continuous carbon, were used as specimen supports for TEM imaging. Grids were prepared as previously described [6]. In order to concentrate the bacteriophages onto the surface of the grid, ultracentrifugation was used following the protocol described in Bergh et al. [1]. Each grid was placed in a centrifuge tube, carbon side up, and 200 µL of seawater or sewage water was added. The tube was spun at 47,000 rpm (100,000 ×g) at a temperature of 4°C for 90 minutes. In order to remove larger entities after centrifugation, the grid was removed from the centrifuge tube and washed with three 200-µL beads of water set up on the surface of Parafilm. Once the grids were washed, they were each stained with 3 µL of 1% uranyl acetate. After 30–60

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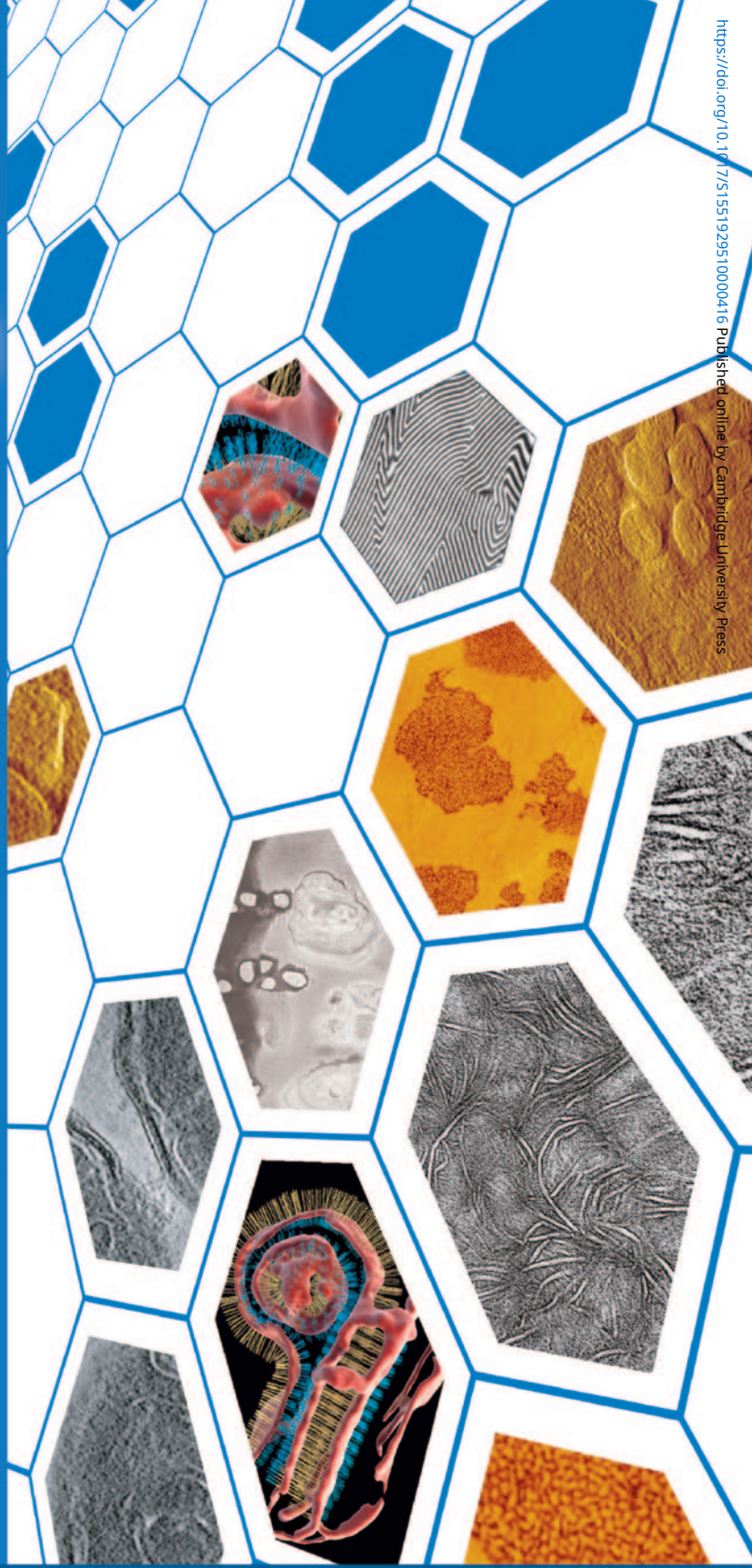

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seconds, excess stain solution was blotted with filter paper, and the grid was left to air dry.

Electron Microscopy. A Tecnai G-2 Spirit (FEI Co.) transmission electron microscope equipped with a 2K × 2K CCD camera (TVIPS F224) was used to examine the grids. Each grid was initially observed at a low magnification (~500×), to identify the well-stained areas. These areas were then searched at higher magnification (~11,500×) to locate the phages. A digital image was then acquired using Leginon software [7] at a range of magnifications (30,000×–67,000×), depending on the size of the phage.

Isolation of Bacteriophages. In order to propagate phages from the collected samples, bacteria were cultured and infected with phages. Four bacterial cultures were established: two with a K12-derived strain of *E. coli* (DH5α), one with bacteria from Mission Beach, and one with bacteria from sewage. Ten milliliters of Luria broth (LB) were inoculated with 10 µL of *E. coli* from a glycerol stock, or the unfiltered concentrated water samples, using a sterile pipette and were incubated overnight at 37°C, shaking at 250 rpm. The following day, one overnight culture of *E. coli* and the overnight culture of sewage bacteria were each infected with 1.6 mL of filtered sewage, which had been concentrated from ~100 mL to 3.2 mL in an Amicon Ultrafree device with a 100-kDa molecular weight cutoff. Similarly, the second overnight culture of *E. coli* and the culture of Mission Beach bacteria were each infected with ~800 µL of Mission Beach water, which had been concentrated from 200 mL to 1.6 mL, then spun at maximum speed in a microcentrifuge to remove bacteria and large particles. All four cultures were incubated for 15 minutes at 37°C without shaking to allow the phages to attach to the bacteria. The inoculated 10-mL cultures were then transferred into 50 mL of prewarmed LB and incubated at 37°C with shaking at 250 rpm. The optical density at 600 nm was checked every 2 hours to monitor bacterial growth and lysis. The bacterial cultures all became saturated at an OD of ~2.0. After 8 hours the *E. coli* infected with sewage phages had an OD of 0.25, showing that the cells had lysed. The lysed culture was stored at 4°C overnight. The culture was spun at 10,000 ×g for 15 minutes to remove any remaining bacteria and cellular debris, filtered through a 0.45-µm filter, and concentrated to a final volume of 250 µL.

Results

Comparison of Water Samples. The raw filtered sewage water (influent) contained a variety of phages, as well as a high background of other organic material (Figure 1). The reclaimed sewage water had a lower background, but the number and variety of phages remained high (Figure 2), with a number of different capsid sizes and with both contractile and non-contractile tails.

Reclaimed water collected from the source at the sewage plant was compared with reclaimed water from a tap at The Scripps Research Institute. Both samples were collected in sterile tubes; neither was filtered to remove bacteria or other large microbes. The reclaimed water from Scripps had bacteria and organic matter that were not observed in the reclaimed water from the sewage plant (Figure 3), suggesting that the water accumulated this material during transportation.

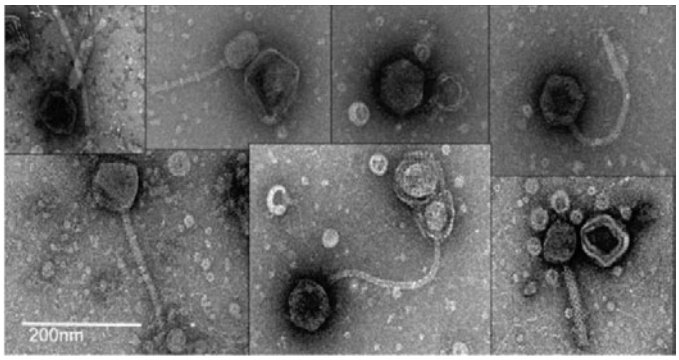

Figure 1: Phages in negatively stained samples of filtered raw sewage water, taken at 30,000× magnification.

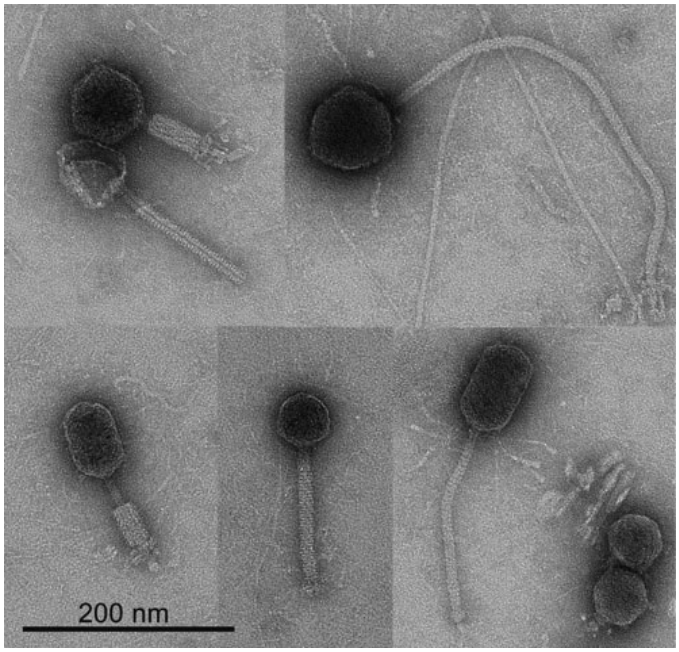

Figure 2: Phages in negatively stained samples of filtered reclaimed water, taken at 52,000× magnification.

The water collected at Mission Beach from the Pacific Ocean also contained a wide variety of phages, algae scales, and bacteria (Figure 4). Interestingly, a bacterium was observed with a large number of phages on its surface, likely caught in the process of infecting the cell (Figure 4B).

Bacteriophage Isolation. Of the four conditions used to isolate phages from the water samples, one phage infection was established. The K–12 *E. coli* were successfully infected by the sewage phages, but not the Mission Beach phages. No phages propagated in the bacteria cultured from sewage or Mission Beach water. There could be many different reasons for this outcome. Some of the possible explanations are that the strains of bacteria collected from the sewage plant and Mission Beach that multiplied best in LB were resistant to the phages or that a larger number of phages would be required to establish a robust infection. TEM images indicate the bacteriophages that were successfully propagated appeared to be all the same, with a long, flexible, non-contractile tail. They also exhibited an isometric head with a diameter of

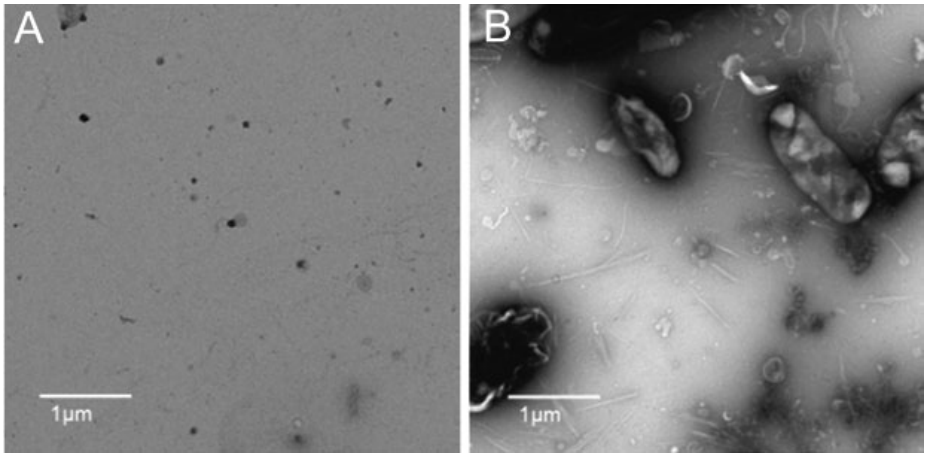

Figure 3: Reclaimed sewage water from the sewage treatment plant (A) compared with reclaimed sewage water collected at The Scripps Research Institute, (B) both in negative stain at 6,500× magnification.

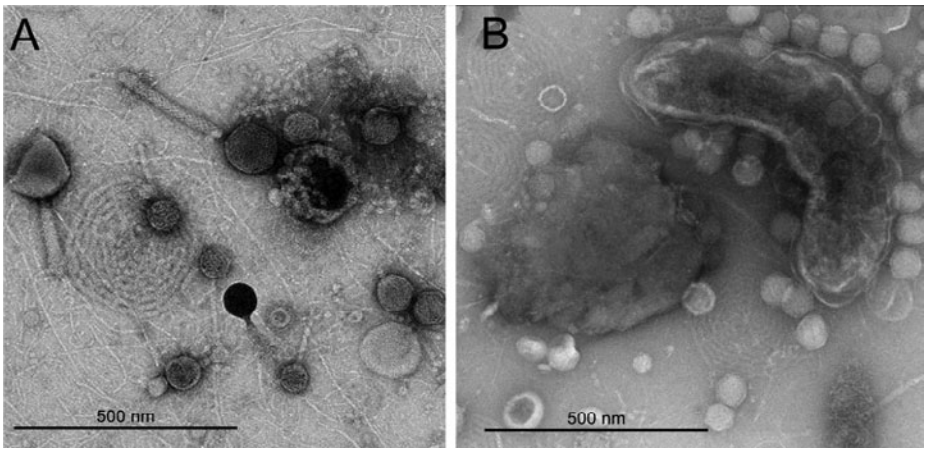

Figure 4: Mission Beach water, unfiltered, both taken at 29,000× magnification. A) Phage and algae scales. B) A bacterium being infected by many phages.

~65 nm diameter (Figure 5). These structural elements are characteristic of phages in the Sipheroviridae family, such as bacteriophage lambda and HK97. Therefore, we tentatively identify the phages propagated in the laboratory from sewage water as members of the Sipheroviridae.

Discussion

Based on observations of various water samples in the electron microscope, the treatment of raw sewage appears quite thorough. Most of the bacteria and other organic material observed in the raw sewage sample (Figure 1) are removed during sewage treatment and are no longer observed in the reclaimed sample (Figure 2). However, phages and potentially other viruses survive the water treatment and are still abundant in reclaimed water (Figure 2). Further testing could determine whether these phages remain infectious in reclaimed water and whether there are any potential medical or ecological implications for their presence in treated water sources.

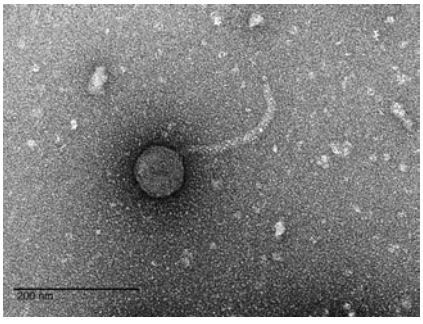

Figure 5: A phage propagated from raw sewage in laboratory *E. coli*; all the phages observed from the culture were of the Sipheroviridae family.

Summary

This project allowed a high school student to investigate the diversity of bacteriophages in various water samples and to learn about the process of phage infection and propagation with host bacteria. The Scripps Research Institute High School Research Education program provides students with the opportunity to learn what it is like to be a scientific researcher. The student had access to modern equipment not found in high school science classes and learned to prepare samples and use an electron microscope.

Acknowledgments

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