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Environmental Sampling of Photosynthetic Microbes and Their Viruses: From Field to Lab

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Abstract

Environmental sampling of photosynthetic microorganisms and their viruses plays a critical role in understanding contemporary marine and freshwater biodiversity and ecosystem dynamics as well as the impacts of climate change-related factors (e.g., rising temperatures and acidification) on evolutionary trajectories of species and community composition. Unfortunately, the diversity of the virosphere does not support a single universal sampling and experimental workflow. Indeed, each virus system has unique features, which require modifications to standard protocols in virology to accomplish research goals. Although virus discovery and characterization require approaches that are specific to the target system, for all viruses, the research aims are similar: isolate the virus; determine host range; confirm productive infection; and characterize the virus, the host, and virus-host dynamics. Robust descriptions of virus-host systems consist minimally of elucidating morphology, physiology, biochemistry, and omics profiles. Further information may be obtained by manipulating the system by changing factors such as multiplicity of infection, temperature, pH, host-switch, directed evolution, or applying drugs to observe virus-host system response. Our laboratory studies viruses across domains of life (Archaea, Bacteria, and Eukarya). In this report, we detail methods for sampling photosynthetic microbes from the euphotic zone of freshwater and marine environments with focus on isolating bacteriophage (i.e., cyanophage) of cyanobacteria. Cyanobacteria are keystone species critical to primary production and nutrient cycling in these aquatic ecosystems. The described workflow extends from sampling waters at different depths to characterizing virus-host system features using liquid and solid media culture, advanced molecular/genetic methods, and analytical approaches. The methods described are adaptable to bacteriophage and virus discovery in virus-host systems across domains of life.

Introduction

Characterizing infection dynamics and evolutionary relationships of cyanophages and their hosts is key to understanding the current and future states of marine and freshwater ecosystems. Advanced high-throughput sequencing techniques and modern analytical methods allow rapid sequencing of cyanophage (and host) genomes to study virus-host relationships[1,2]. However, workflows begin with environmental sampling to isolate and identify viruses and their hosts[3,4,5]. Due to the diversity of the virosphere, there is no single protocol for sampling and characterizing all viruses and bacteriophages. Indeed, each virus-host system has its unique characteristics. Nonetheless, the baseline objectives are similar regardless of the virus system being explored: isolate the virus; determine host range; demonstrate productive infection in one or more hosts; characterize the virus, host, and virus-host infection dynamics using qualitative and quantitative approaches. An advanced understanding of the virus system can be examined by perturbing the system. Changing parameters such as multiplicity of infection (MOI)[6], temperature, pH, the host strain, or by the application of drugs to determine how the system responds, provides additional insight into the nature of virus-host dynamics. More advanced manipulation, such as directed evolution, can provide additional information about the potential evolutionary trajectory of the system and how co-evolutionary endpoints may impact the larger microbial community and ecosystem.

In this study, we present a model (but readily adaptable) protocol for environmental sampling of photosynthetic microbes such as cyanobacteria and microalgae, which play a critical role in nutrient cycling and primary production in marine, freshwater, and other aquatic ecosystems[7,8,9,10,11]. The workflow focuses on isolating hosts from the euphotic zones of marine and freshwater environments and their viruses and bacteriophages, which are key drivers in these ecosystems[12]. Both viruses and bacteriophages impact both eukaryotic and prokaryotic population structures and heavily influence ecosystem diversity and productivity[13]. Thus, the goal here is to provide a sampling protocol and post-sampling workflow that facilitates enrichment of hosts and viruses for investigating: single-virus/single-host, multi-virus/single-host, single-virus/multi-host, and multi-virus/multi-host dynamics; -omics substrates underlying virus-host infection dynamics; and, potential co-evolutionary trajectories and evolutionary endpoints that impact the virus-host system under study and the larger ecosystem from which the viruses and host were extracted.

Specifically, we describe a workflow for environmental sampling, field pre-processing of samples (i.e., before reaching the lab), post-processing for isolation of bacteriophage (and viruses), enrichment of mixed-species cultures of microorganisms towards identifying permissive hosts, developing single-colony isolates of putative hosts, confirming a *bona fide* virus-host relationship (i.e., productive infection), and characterizing fundamental infection properties (e.g., virulence). Infection assays not only permit calculation of relative virulence (V_R)[14] and host resilience (R_R)[15] but also include steps for extracting culture samples at key time-points for investigating -omics substrates that underlie the observed physiology (i.e., the virus-host interaction dynamics). Lastly, we discuss potential pitfalls, troubleshooting, and solutions, as well as ways to modify select steps to target specific taxa. The workflow is applicable to cyanophage-

cyanobacteria, phycodnavirus-microalgae, and thermophilic fusellovirus-archaeal systems[16,17], demonstrating its utility in characterizing viruses across all three domains of life: Bacteria, Eukarya, and Archaea.

Protocol

1. Environmental sampling

NOTE: This protocol describes offshore sampling for photosynthetic microbes from the euphotic zone within 5 miles of the shoreline off the coast of southern California. However, the protocol has been adapted to sample from lakes, frozen lakes, or geothermal hot springs (**Figure 1**).

1. Environmental sampling offshore along the southern California coastline

   NOTE: A 6 m, deep-V, center console vessel (i.e., boat) with dual 150 hp outboard motors was loaded with research equipment (**Table of Materials**). Three sites were selected for euphotic zone sampling: (a) Site 1: N 30° 15.436, W 117° 31.551; (b) Site 2: N 33° 12.521, W 117° 25.275; and (c) Site 3: N 32° 59.914, W 117° 17.058. At each site, 20 L of water samples were extracted midday from three different depths at each site: < 3 m (surface sample), 6 m, and 14 m.

   1. Transfer pump assembly: Upon reaching each sample site, deploy the boat anchor and record GPS coordinates from the navigation system. Assemble the sampling system.

      1. In brief, fit one end of 2.54 cm I.D. flex-plus blue PVC tubing (50 ft.) to a 2.54 cm suction strainer to prevent large debris from entering the pump system. Secure the strainer-tube junction with duct tape. Fit the opposite end of the tube to the intake manifold of the 1 hp stainless steel transfer pump.

      2. Attach a 10 ft section of 2.54 cm I.D. collapse-resistant rubber flex hose to the outlet of the pump. Plug the transfer pump electrical cord into the gas-powered 3800 W inverter generator. Once assembled, prime the transfer pump and test to ensure the pump system is operational (**Figure 1**, top row).

   2. Weighting the transfer tube: Secure one end of a 25 m long, 1.25 cm diameter, nylon marine rope to the eyelet of a 14 kg mushroom anchor. Use duct tape to secure the rope to the blue PVC tubing every 3 m with the suction strainer set approximately 1 m above the anchor to prevent the strainer from lodging into the sand or hitting rocks underwater. Use the anchor to submerge the transfer tube (blue PVC tube) and to avoid any strong undercurrents from creating a large angle between the boat and the tube, which would give inaccurate sample depths. Lower the anchor/rope and tube assembly to depth.

   3. Extracting seawater samples: Once the tube tip with the strainer is at the desired depth, power up the generator and pump. Reprime the pump or shake the transfer tube to get water to start pumping from depth. Once a steady flow of water was being transferred from depth, discharge 1-2 tube volumes back into the ocean to ensure that water from the desired depth is flowing through the pump. Collect water samples in 20 L water jugs (**Figure 1**, top row, far right).

   4. Sample transport to field station: Fill 20 L water jugs to approximately 18 L to allow gas exchange (e.g.,

O_2/CO_2) between water samples and headspace during transport. Prior to capping water jugs, record temperature, pH, and salinity from raw water samples. Then, cap and place water jugs on an ice bed in sub-deck storage and cover with a light sheet to avoid over-exposure to sunlight and high temperatures.

2. Field pre-processing of water samples

NOTE: Upon arrival at an onshore facility (e.g., hotel room/conference room/field station), a series of pre-processing steps were undertaken to protect microbial content within the water samples and to separate different fractions of the seawater samples based on particle size.

1. Filtration system assembly: Secure one end of a 1 m long piece of 9.525 mm I.D. clear vinyl tubing to the hose barb inlet on the vacuum port of a portable air diaphragm vacuum pump. Secure the opposite end of the same hose to a 1.0 L, 0.45 µm filter vacuum cup assembly. Use both 0.45 µm and 0.22 µm filter cup assemblies in sequence to separate different fractions of the seawater sample as described below.

2. To test the system for leaks or defective/torn filters, filter 0.25 L of seawater through the 0.45 µm polyethersulfone (PES) membrane filter cup. A non-leaky system will show suction between the filter and the grating once the seawater sample is completely filtered through.

3. Filtration through 0.45 µm PES membrane filter cup assembly: To isolate photosynthetic microorganisms (e.g., microalgae and cyanobacteria), the goal is to filter 2 L of seawater sample through the 0.45 µm PES membrane filter cup using two 1 L filter cups (or four 500 mL filter cups). Appropriately label all filtrate bottles with filter size, depth from which the sample was taken, date, and sampling site designator. Finally, screw cap and cover bottles containing 0.45 µm filtrate with a semi-transparent cloth or sleeve to prevent excessive sunlight.

NOTE: Do not allow the filter in the filter cup to run dry. Always keep a thin layer of liquid on the filter. The filter will accumulate biological debris (e.g., cells). If the filter becomes clogged with debris, carefully unscrew the cup assembly and set the used filter cup aside, but do not discard the clogged cup. Attach a new 0.45 µm filter and continue.

4. Filtration through 0.22 µm PES membrane filter cup assembly: To isolate large eukaryotic viruses, use 1 L of the 0.45 µm filtrate from the previous step (step 1.2.3) and filter through a 0.22 µm filter cup. Appropriately label the ultrafiltrate bottle with filter size, depth from which the sample was taken, date, and sampling site designator. Finally, screw cap and cover the bottle containing 0.22 µm ultrafiltrate (from filtering the 0.45 µm filtrate).

NOTE: Do not allow the filter in the filter cup to run dry. Always keep a thin layer of liquid on the filter. The filter will accumulate biological debris, which contains biological samples. If the filter becomes clogged, unscrew the cup assembly and set aside the used filter cup. Do not discard the clogged cup. Attach a new 0.22 µm filter and continue.

5. Concentrate biomass from 0.45 µm filter: Pour 48 mL of filtrate (from 0.22 µm filtration) into each of two sterile 50 mL conical polypropylene centrifuge

tubes. Use a sterile scalpel to uniformly cut along the outermost edge of the expended 0.45 µm filter within the cup assembly. Use sterile forceps to remove the biomass-laden and moist filter. Cut it in half. Roll up half of the filter paper into one 50 mL conical tube. Repeat with the second half of the filter membrane and the second tube. Screw on tube caps and secure with transparent film. Label the tube.

6. Concentrate large virus biomass from 0.22 µm filter: Pour 14 mL of ultrafiltrate into each of two sterile 15 mL conical polypropylene centrifuge tubes. Use a sterile scalpel to cut along the outer rim of the used 0.22 µm filter within the cup assembly. Use sterile forceps to remove the biomass-laden and moist filter. Cut it in half. Roll up and insert one half of the filter paper into one of the 15 mL conical tubes. Repeat for the second half of the filter membrane and the second 15 mL conical tube. Screw on tube caps and secure with transparent film (e.g., Parafilm). Label each tube with filter size (i.e., 0.22 µm), site, depth, and date.

7. Retain the remaining 0.45 µm filtrate as well as the remaining 0.22 µm filtrate for shipment back to the lab. Repeat this entire filtration process (steps 1.2.2 through 1.2.6) for each depth and sampling site.

   NOTE: Ultrafiltrate is used in the lab as a media base (with native minerals and other nutrients).

   Raw sample microscopy (Optional): Take one 50 mL conical tube with a 0.45 µm filter in it and gently shake. Use a plastic dropper (or pipettor) to take 30-50 mL of seawater from the 50 mL conical tube and prepare a glass microscope slide with the sample and a cover slip. Using a field microscope and a computer, view the microscope slide content to detect and record characteristics of observed microorganisms (**Figure 2D**).

2. Sample processing to concentrate viruses/ bacteriophage and enrich hosts

NOTE: This protocol describes the first processing steps required once samples reach the laboratory from the field site. Transport by airplane versus road vehicle may require different considerations. Environmental sample viability will decrease with abrupt changes in temperature, pH, salinity, exposure or lack of exposure to light, dissolved gas (e.g., CO_2 and O_2) concentration, and time between environmental extraction and lab processing.

1. Spin concentrating and storing viruses and bacteriophages

   NOTE: Once samples arrive at the lab, processing within 8 h after arrival may minimize loss of viable microbes due to the time lapse between sample site extraction and arrival at the lab. The steps provided below will provide aliquots of concentrated bacteriophage (or virus) suspension (i.e., working stocks) for long-term storage and use.

   1. Concentrating ultrafiltrate: For each sampling depth by site, ship the remaining ~800 mL of ultrafiltrate to the lab. Use a 3K NMWL 15 mL centrifugal spin filter and centrifuge at 6000 x *g* for 10-20 min to concentrate 50-100 mL of ultrafiltrate down to 1.0-1.5 mL. Prepare grids for TEM imaging as previously described[16] to assess whether there are any virus-like particles (VLPs) in the resulting concentrated ultrafiltrate. If VLPs are detected, divide the concentrate into 100 mL aliquots. Store at 4 °C (or -20 °C for longer-term storage).

2. Concentrating particles from 0.22 µm filter: Use a sterile scalpel to gently scrape debris from the 0.22 µm filters into the ultrafiltrate within the 50 mL tubes in which filters were transported. Use a 10K NMWL spin-concentrator cup assembly to concentrate 50-200 mL of suspension down to a volume of ~3 mL of sample retentate. Prepare grids for TEM imaging as previously described[16] to determine if there are VLPs from the 0.45 µm filtrate that may have been captured on the surface of the 0.22 µm filter. If VLPs are detected, divide the concentrate into 100 mL aliquots and store at 4 °C (or -20 °C for longer-term storage).

2. Spin concentrating and enriching retentate from 0.45 µm filters.

NOTE: Mixed cultures of microorganisms may contain hosts for the viruses and bacteriophage isolated above (steps 2.1.1 and 2.1.2). Spin concentration and enrichment of mixed cultures provide working stocks from which to develop pure cultures of putative hosts (see step 3).

1. Concentrating mixed suspensions: For each sampling depth by site, ship back ~100 mL of ultrafiltrate with a cell debris-laden 0.45 µm filter to the laboratory. Use a sterile scalpel to scrape debris from the 0.45 µm filter into the ultrafiltrate in the 50 mL tubes in which they were transported. Centrifuge the sample at 2000 x *g* for 10 min, 4 °C to pellet the biomass. Resuspend pellet in 5 mL of 1:8 dilute BG-11 media[18] by gentle trituration with a pipette.

2. Enriching retentate from 0.45 µm filter: Prepare six parallel cultures in 250 mL baffled Erlenmeyer flasks containing: 50 mL of BG-11 media (3 flasks) and a mix of ultrafiltrate from the sample site (40 mL) plus BG-11 (10 mL). Inoculate each flask with 0.5 mL of cell concentrate (from step 2.2.1). Incubate with mild shaking in a photobioreactor for a minimum of 7-10 days at 25 °C with light intensity set at 20-40 mmol photons/m^2/s.[19] Once growth is robust (e.g., OD_{730nm} = 0.8), centrifuge at 2000 x *g*, 10 min, 4 °C to pellet. Resuspend the pellet in 10 mL of 1:8 dilute BG-11 media by gentle trituration with a pipette. Prepare 10-20 aliquots of 0.5 mL of cell suspension in screw-cap cryotubes for -80 °C storage.

3. Developing single-genotype pure cultures of microbial hosts

NOTE: This protocol describes procedures for isolating and purifying putative microbial hosts for viruses and bacteriophage from mixed cultures processed from collected environmental samples. Multiple host phenotypes may be isolated and purified, thus increasing the probability of detecting permissive hosts for the viruses and bacteriophage present in stored water samples (steps 2.1.1-2.1.2).

1. Streaking plates and selecting single-colony isolates

NOTE: Multiple single-genotype pure cultures are developed from mixed cultures to increase the probability of isolating a susceptible and permissive host of viruses or bacteriophage captured during environmental sampling and to investigate the host range of viruses and bacteriophage.

1. Develop single colony isolates (SCI) as described below.

1. Prepare BG-11 agar plates[20]. Thaw on ice, one aliquot of the mixed suspension which was stored at -80 °C (from step 2.2.2). Use

a sterile loop to streak cell concentrates onto the BG-11 agar plates using standard techniques[20]. Incubate streaked plates in a photobioreactor for ~10 days at 25 °C with light intensity at 20-40 mmol photon/m^2/s or until colonies appear on plates.

2. Prepare ~10 seed cultures in 20 mL culture tubes with 5 mL of BG-11 media and/or 5 mL of a mix of ultrafiltrate from the sample site and BG-11. Pick a SCI from the plate with a sterile toothpick and inoculate each tube individually by dropping one toothpick into each tube. Incubate with mild shaking in a photobioreactor for a minimum of 10-15 days at 25 °C with light intensity set at 20-40 mmol photon/m^2/s or until growth is detected (e.g., OD_{730nm} = 0.4-0.8).

   NOTE: If there are multiple colony phenotypes (e.g., morphology, transparency, color) on the first streak plate, select individual colonies for inoculation from a range of colony phenotypes.

2. Developing pure cultures of working stocks: Once positive seed culture growth is detected, repeat streak procedures until only a single colony phenotype appears on plates (**Figure 3B** and **Figure 4B**). Upscale seed cultures to 50 mL cultures in 250 mL baffled flasks (**Figures 3C** and **Figure 4C**) as described above (step 2.2.2). Prepare ~20 aliquots of 0.5 mL of SCI cell suspension and pipette into 1.0 mL screwcap cryotubes for -80 °C storage.

   NOTE: For developing pure cultures of cyanobacteria, antibiotics or antifungals may be required (e.g., imipenem, cycloheximide) to reduce culture contamination[21]. The plating procedure is a pivot point where isolation of cyanobacteria or microalgae may be targeted. Light parameters during incubation may vary in terms of light intensity or on-off cycles.

2. Single genotype characterization and storing working stocks

   NOTE: To confirm the isolation and enrichment of individual putative host genotypes, pure cultures are characterized morphologically and genetically.

   1. Morphological characterization: Once cycles of liquid culture and solid media plate work result in a single cell phenotype, characterize microbial morphotypes by both light microscopy (**Figure 2A-C**) and, if available, transmission electron microscopy (TEM) and/or scanning electron microscopy (SEM; **Figure 3A** and **Figure 4A**). Under light microscopy (e.g., 60x-100x magnification) green, light brown, or transparent unicellular, colonial, filamentous, spiral, branched, or trichome cellular organization may be observed. This will indicate a possible taxonomic assignment.

   2. Genetic Characterization: Validate the isolation of a single microorganismal genotype by DNA extraction from the putative pure cultures, followed by partial genome sequencing. In cyanobacteria, the 16S rRNA gene is often a target (**Figure 5A**)[22]. A 16S phylogenetic analysis can identify taxonomic grouping[22]. In microalgae, 18S rRNA is often targeted[23]. For isolates of interest or novel isolates, whole genome sequencing may be pursued.

4. Developing single-genotype virus/ bacteriophage suspensions

NOTE: This protocol describes procedures for isolating and purifying multiple single-genotype viral and bacteriophage suspensions from environmental samples.

1. Dilution and TEM of VLP concentrates

   NOTE: Using TEM and plate-based plaque assays[24], confirmation that VLPs are bona fide bacteriophage (or viruses) that infect host cells is achieved.

   1. Transmission electron microscopy of filtrates (0.45 µm and 0.22 µm): Use concentrates from 0.45 µm and 0.22 µm filtration of raw water samples (see step 2.1) to prepare TEM grids[16] and image to examine virus particle heterogeneity (i.e., diversity of morphotypes). Dilute samples that result in grids with high confluency (e.g., 90%) before completing subsequent steps to achieve 30%-50% confluency. Manual particle counting or the use of imaging software may be employed to estimate cell confluency on grids.

      NOTE: High confluency on grids can result in clearing of host lawns on solid media plates rather than emergence of individual plaques that may be selected. Thus, diluting samples prior to plaque assays may save time. Alternatively, multiple host lawn plates may be tested for host-virus interactions using serial dilutions of VLP concentrates.

   2. Detecting bacteriophage (or virus) activity on host lawns: On BG-11 agar plates, grow putative host lawns of cyanobacteria (or microalgae) from working stocks (step 3.1.2). Pipette 0.5 mL of VLP suspension in 4.5 mL of media onto lawns (**Figure 6A**, left) and gently swirl until the entire surface of the lawn is coated with suspension. Incubate in a photobioreactor for 3-10 days at 25 °C with light intensity at 20-40 mmol photons/m^2/s or until plaques are visible on plates (**Figure 6A**, right).

2. Scraping plaques and developing single genotype virus or bacteriophage suspensions

   NOTE: By scraping individual plaque phenotypes and propagating bacteriophage in liquid cultures of the host, bona fide host-bacteriophage (or host-virus) interactions are confirmed, permitting bacteriophage (or virus) working stocks to be developed for infection assays. Working stocks of bacteriophage (or virus) must be titered by one of several methods (e.g., qPCR, plaque assays) to proceed with infection assays at a defined multiplicity of infection (MOI).

   1. Isolating and propagating one bacteriophage (or virus) genotype: Once individual plaques can be seen on plates (due to adequate dilution of VLP concentrate), use a sterile loop to scrape a single plaque. Swirl the loop in a 10 mL seed culture of the host (**Figure 6B**, left). Incubate with mild shaking (e.g., 70-120 rpm) in a photobioreactor for 3-10 days at 25 °C with light intensity set at 20-40 mmol photon/ m^2/s or until OD_{730nm} drops or cell debris is detected in the bottom of the tube, which indicates cell death/ lysis (**Figure 6B**, right)[25].

      NOTE: If plaques form on host lawns but there are no signs of infection in liquid culture, plaques may have been formed by a bacteriocin[26] rather than a bacteriophage (or virus). TEM can support plate assays by indicating cell death by bacteriophage (or viral) infection.

2. Quantifying titer for bacteriophage (or virus) working stocks: Bacteriophage (or virus) isolation is achieved using the filtration and spin-concentration methods described above. Quantify titers for bacteriophage (or virus) suspensions by serial dilution plaque assays[27]. Alternatively, perform DNA extraction and whole virus genome sequencing and phylogenetics (**Figure 5B**) to develop primers for conducting qPCR-based titering of bacteriophage to establish working stocks for subsequent infection assays.

   NOTE: Propagation of single bacteriophage (or virus) genotypes may be upscaled to 50 mL or 100 mL cultures if titer or working stock volumes are too low for experimental needs. Consider the nature of the virus-host system (e.g., high virulence or low virulence), the multiplicity of infection (MOI) that is planned for infection assays, and how many infection assays will be completed for a project when developing working stocks of bacteriophage (or viruses). It is not advisable to thaw and re-freeze virus stocks. Thaw and use one full virus stock from replicates of the same preparation at the same titer for each experiment. Other titer techniques may complement the methods described, including electrospray ionization/mass spectrometry (ESI/MS)[28].

5. Infection assays

NOTE: This protocol describes host-phage (or host-virus) infection assays that are used to: qualitatively characterize virulence, host range, and infection phenotype (an indicator of replication strategy) on solid media; and quantitatively assess growth kinetics, virus virulence[14], and host resilience[15] in liquid culture.

1. Determining relative bacteriophage (or virus) virulence and host range on solid media

   NOTE: All solid media assays, including spot-on-lawn assays, single-plate serial dilution plaque assays, and host resistance plate assays, provide qualitative data about bacteriophage-host infection parameters such as virus virulence, host range, and replication strategy. Liquid culture growth assays allow for more quantitative measures and modeling of virus-host dynamics.

   1. Spot-on-lawn assays: To determine host range, prepare lawns of multiple putative hosts isolated from environmental samples or from strains acquired from other labs or culture collections. On lawns, spot 2 µL of bacteriophage (or virus) suspension on the lawn with a pipette. On a separate region of the same plate, spot the same volume of Triton X-100 and nanopure (18MW) water as positive and negative controls, respectively[16]. Conduct multiple spot-on-lawn assays with a given bacteriophage (or virus) on multiple hosts to assess the host range of the bacteriophage[16]. Plaque phenotypes offer insights into the bacteriophage (or virus) replication strategy. Plaques that have sharp borders and that clear down to the agar indicate lytic replication, while plaques with diffuse borders or a halo may indicate non-lytic replication[16,17].

      NOTE: Alternatively, single-plate serial dilution plaque assays indicate whether there is a linear relationship between plaque size and bacteriophage titer. Use a marker on the back side of the plate to partition the plate into a grid or pie sections. Then,

using the same volume (i.e., 2 mL) but with dilutions of bacteriophage suspension (e.g., 1:1, 1:4, 1:16, 1:64), conduct the spot-on-lawn assay on a single host plate.

2. Host resistance assays: To determine whether a suite of bacteriophages may produce infection on a given host, prepare a host lawn on a plate as described above. Using a marker on the back side of the plate, partition the plate into a grid or pie sections. Then, spot 2 mL of each bacteriophage (or virus) suspension into one section of the grid (or pie), using Triton X-100 and nanopure water as controls. If all bacteriophage (or virus) suspensions are added at equal titers, then assess relative virulence between bacteriophage strains (or viruses) on host qualitatively (i.e., larger plaque = more virulent virus).

2. Quantifying virulence, resilience, and growth in liquid culture infection assays

NOTE: Initial qualitative results provide a basis for targeting specific bacteriophage-host pairs and conducting more quantitative analyses of virus-host interactions dynamics. In liquid culture, an infected-host growth curve is generated and compared to uninfected (control) host growth.

1. Perform liquid culture single-virus/single-host infection assay as described below.

1. Establish parallel liquid cultures (~6-12) of a microorganism (e.g., a cyanobacterium) proven to be susceptible and permissive by solid media plate assays (step 5.1) in either: 1.0 L (320 mL of culture), 500 mL (160 mL of culture), or 250 mL (80 mL of culture) - baffled culture flasks, as previously described[17]. At a pre-defined time point (typically 0.5 or 1 host doubling time), inoculate a subset of parallel cultures at equivalent cell density (e.g., OD_{730nm} = 0.08) in triplicate with bacteriophage suspension using the appropriate volume required to yield a pre-selected multiplicity of infection (MOI) with available working stock titer.

2. Leave other parallel cultures (also in triplicate) un-inoculated (i.e., uninfected) as controls. Optionally, inoculate a third set of parallel cultures with a different bacteriophage (or virus) strain for comparisons between select bacteriophage-host pairings. Allow cultures to grow in a photobioreactor for 3-10 days at 25 °C with light intensity set at 20-40 mmol photons/ m^2/s.

3. Generate host growth curves by taking OD_{730nm} readings at regular time intervals (e.g., 8 h) from bacteriophage-infected and uninfected (control) cultures (**Figure 7A**). Use TEM verification of uninfected control cultures (**Figure 7C**) and productive infection in host cell cultures (**Figure 7D**). Cytopathic effects[29,30] are readily observable in most infected cells, while uninfected cells do not show signs of morphological transformation or stress.

2. Baseline analytics for liquid culture infection assays: From single-virus/single-host (SVSH) liquid culture infection assays, several baseline metrics can be derived, such as: maximum specific growth rate (μ_{max})[31], relative bacteriophage (or virus) virulence[14], and relative host resilience[15]- for a

more quantitative description of bacteriophage-host or virus-host dynamics (**Figure 7B**). Standard reportable metrics include growth rate (often reported as maximum specific growth rate, or μ_{max}) and relative virulence (VR)14 compared to an uninfected host control and/or a separate bacteriophage on the same host. Other metrics such as system carrying capacity ($N_{asymptote}$) and host relative resilience (R_R)[15] may also be reported.

Representative Results

The use of a portable gasoline-powered electric generator, a water transfer pump, and collapse-resistant tubing serves as an effective way to extract water samples from the ocean, lakes, frozen lakes, and other aquatic environments (e.g., lagoons) at select depths (**Figure 1**, top three rows). Alternative equipment is required for high-temperature acid waters of geothermal hot springs, pools, and mud pots, which can feature pH < 4 and temperatures greater than 90 °C. A long bamboo pole or telescoping aluminum swimming pool cleaning pole with a fixture to hold a 250 mL bottle or 50 mL conical tube can be used to extract water samples from these extreme environments (**Figure 1**, bottom row). In all cases, baseline data on water samples are recorded, including temperature, pH, salinity, and GPS coordinates. (**Figure 1**, bottom row, middle left).

Water samples are transported without delay from the sampling site to a field station or work room where sample pre-processing may be completed (see **Figure 8A**, grey box; step 1.2). Verification of living microorganisms is completed during preprocessing using a field microscope (see **Figure 2D**).

After a series of filtration and purification steps (steps 2 and 3), pure cultures of photosynthetic microorganism are viewed under light microscopy (**Figure 2A-C**). Based on morphological features (**Figure 2B**, **Figure 3A,B**) and 16S rRNA sequencing followed by phylogenetic analysis (**Figure 5A**), further processing of isolates focuses on a culture suspected of being a species of cyanobacteria from the family *Merismopediaceae*, perhaps of the genus *Synechocystis*[32]. Although another isolate appears to be a cyanobacterium, perhaps of the genus *Synechococcus*[33] (**Figure 2A** and **Figure 4A**), for this paper we selected the less studied cyanobacteria group (i.e., *Synechocystis*). This isolate is designated as strain SWII. Strain SWII readily forms homogeneous lawns on BG-11 agar plates (**Figure 6A**), which renders the strain amenable to plate-based plaque assays (step 5.1).

Isolation and purification of a bacteriophage that productively infects strain SWII, as evidenced by spot-on-lawn and swirl-on plates (**Figure 6B**), suggests that the isolate is indeed a cyanophage, which is designated φSBL14 (**Figure 5B**). Although φSBL14 infects and impedes the growth of strain SWII in liquid culture (**Figure 6B**, right), a phylogenetic analysis groups φSBL14 with other previously reported cyanophage[34] that infect species of the genus *Synechococcus* (**Figure 5B**). Note that DNA polymerase genes are highly conserved within cyanopodoviruses and thus are a robust target for phylogenetic analysis[35]. However, it is not uncommon for cyanophage to be promiscuous with a broad host range, thereby infecting multiple species within the order *Synechococcales*[36].

Host growth in liquid culture with concentrated φSBL14 suspension (**Figure 7A**, red line) and a 1:2 diluted φSBL14 suspension (**Figure 7A**, blue line) infecting strain SWII is

compared to uninfected (control) SWII growth (**Figure 7A**, black line). The liquid culture infection assays (step 5.2) show significantly different growth dynamics between host-infected growth curves and the uninfected control. Baseline metrics are calculated (**Figure 7B**). For the uninfected control culture, maximum specific growth (μ_{max}) for SWII is 0.3122 AU/day, while μ_{max} for φSBL14-infected SWII with 1:2 diluted bacteriophage suspension is 0.275 AU/day. When bacteriophage suspension is not diluted, a significant impact on host growth is observed with a μ_{max} of 0.139. The relative virulence index values between infections with diluted versus undiluted bacteriophage suspension are 9.59 versus 67.26, respectively (see **Figure 7B** and **Table 1**)

Infection of SWII by φSBL14 is further confirmed by TEM. Uninfected cultures of strain SWII show no signs of cell stress (**Figure 7C**), while φSBL14-infected cultures of strain SWII exhibit cytopathic effects including membrane thickening, irregular granules in the cytoplasm, and potential blebbing of particles from the cell membrane (**Figure 7D**).

These representative results presented in this report describe the isolation and characterization of an unknown cyanobacterium, likely to be a species of the family *Merismopediaceae* and, perhaps, a member of the genus *Synechocytis*, which is shown to be a susceptible and permissive host to a novel cyanophage. This cyanophage is designated φSBL14. It is phylogenetically related to previously described cyanophages of cyanobacterial hosts within the order Synechococcales (**Figure 5B**). Isolation and characterization of this cyanophage-cyanobacterial host system follow the above-described experimental workflow (also see **Figure 8**). This workflow has also been used with only slight modifications to isolate and characterize other types of virus-host systems: bacteriophage and their hosts, microalgal viruses and their hosts, and archaeal viruses and their hosts.

Figure 1: Extracting samples from different aquatic environments using similar procedures. A water transfer pump driven by a portable gasoline-powered electric generator and fitted with collapse-resistant hoses of appropriate length permits the extraction of water samples from defined depths within the euphotic zones of oceans, lakes, and frozen lakes (top three rows). Sampling protocols can be modified for environments not conducive to automated equipment (e.g., pumps) such as geothermal pools and hot springs (bottom row). Upon acquiring samples, the procedures are standardized in terms of taking baseline measures (e.g., pH, temperature, salinity) and location information (i.e., GPS coordinates). Please click here to view a larger version of this figure.

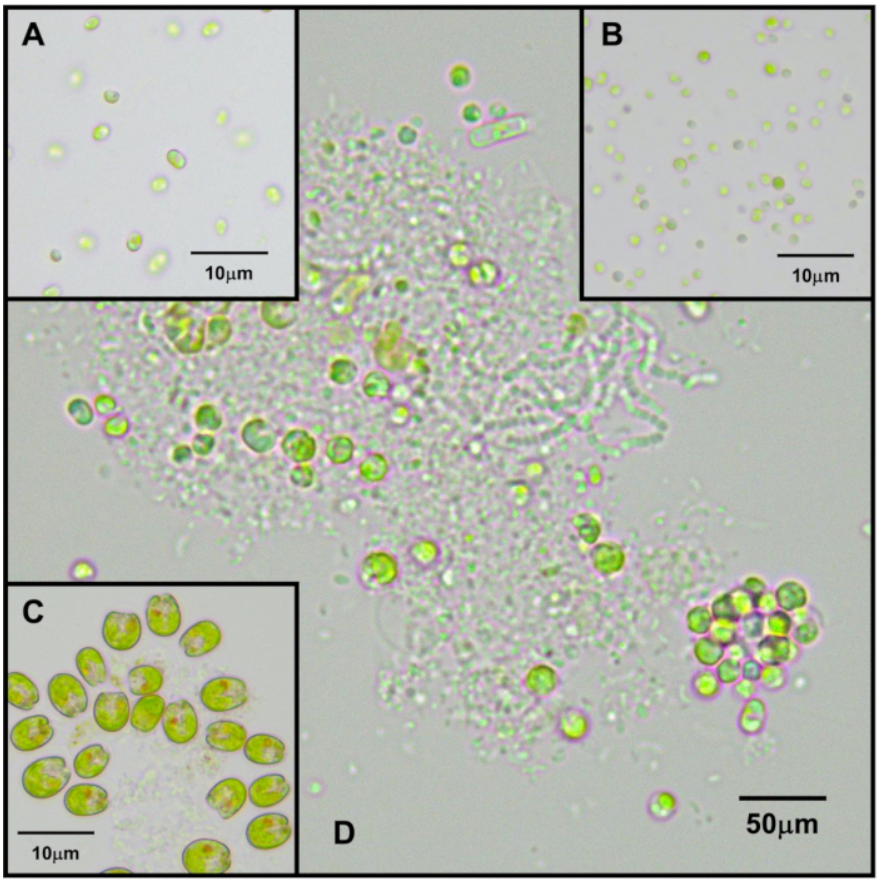

Figure 2: Light microscopy of photosynthetic microorganisms from environmental samples. (**A**) Light microscopy of a photosynthetic microbe derived from culturing a single-colony isolate picked from a streak plate. Based on morphology, the isolate is suspected of being a species of the genus *Synechococcus*. (**B**) Light microscopy of a photosynthetic microbe derived from culturing a single-colony isolate picked from a streak plate. Based on morphology, the isolate is suspected of being a species of the genus *Synechocystis*. (**C**) Light microscopy of a photosynthetic microbe derived from culturing a single-colony isolate picked from a streak plate. Based on morphology, the isolate is a species of unidentified microalgae. (**D**) Light microscopy of the mixed raw water sample from which single-colony isolates were derived. Please click here to view a larger version of this figure.

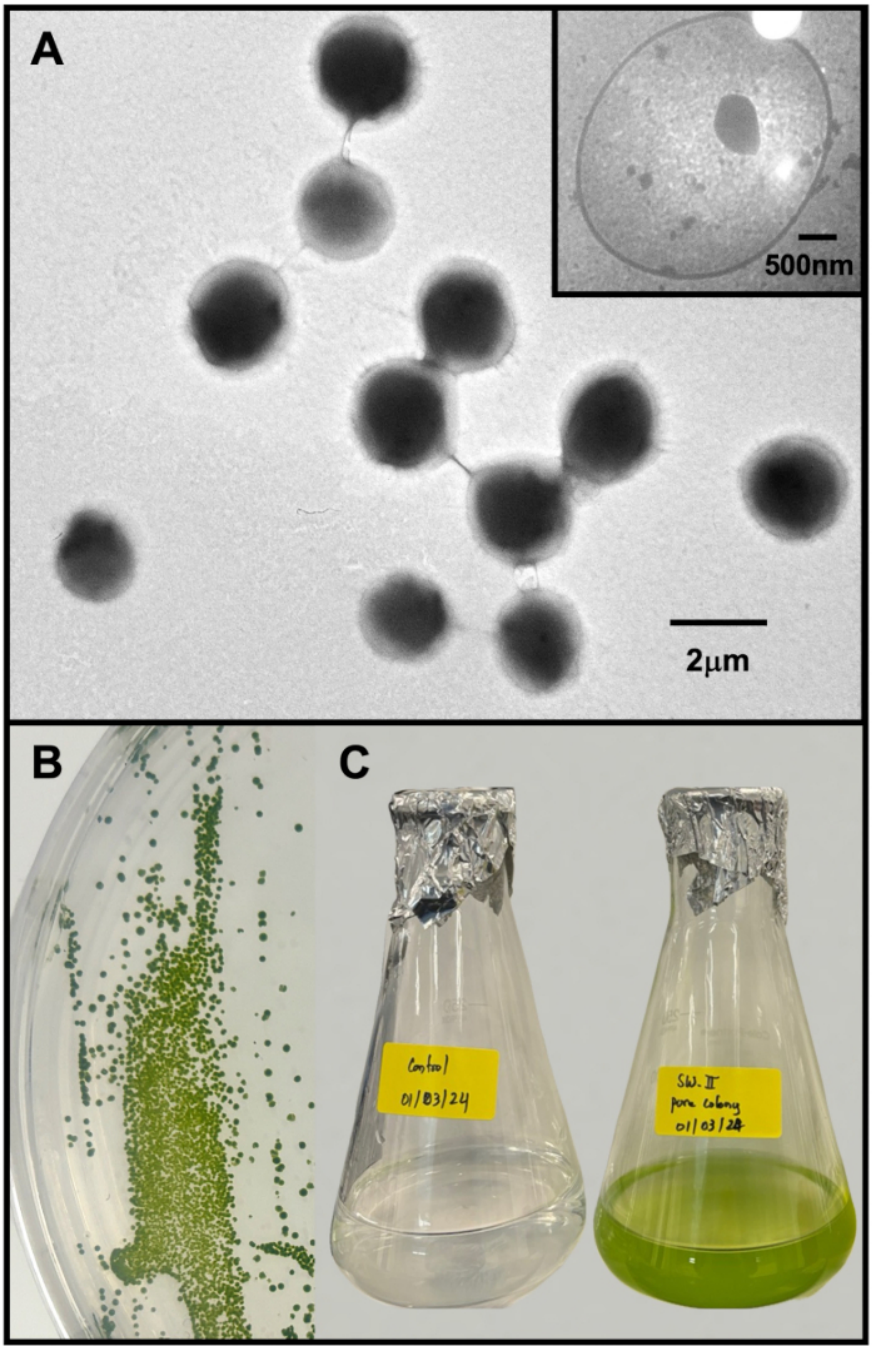

Figure 3: Transmission electron microscopy (TEM) and cultures of unknown strain SWII. (**A**) TEM of a cyanobacterium designated as strain SWII reveals a coccoidal cell of ~2.5-3.0 mm in diameter; isolate SWII is suspected of being a species of *Synechocystis* based on morphology. (**B**) Streak plates show that strain SWII forms large bright green colonies with sharp boundaries on BG-11 agar plates. (**C**) Liquid culture of SWII reveals bright green cell growth in BG-11 media. Please click here to view a larger version of this figure.

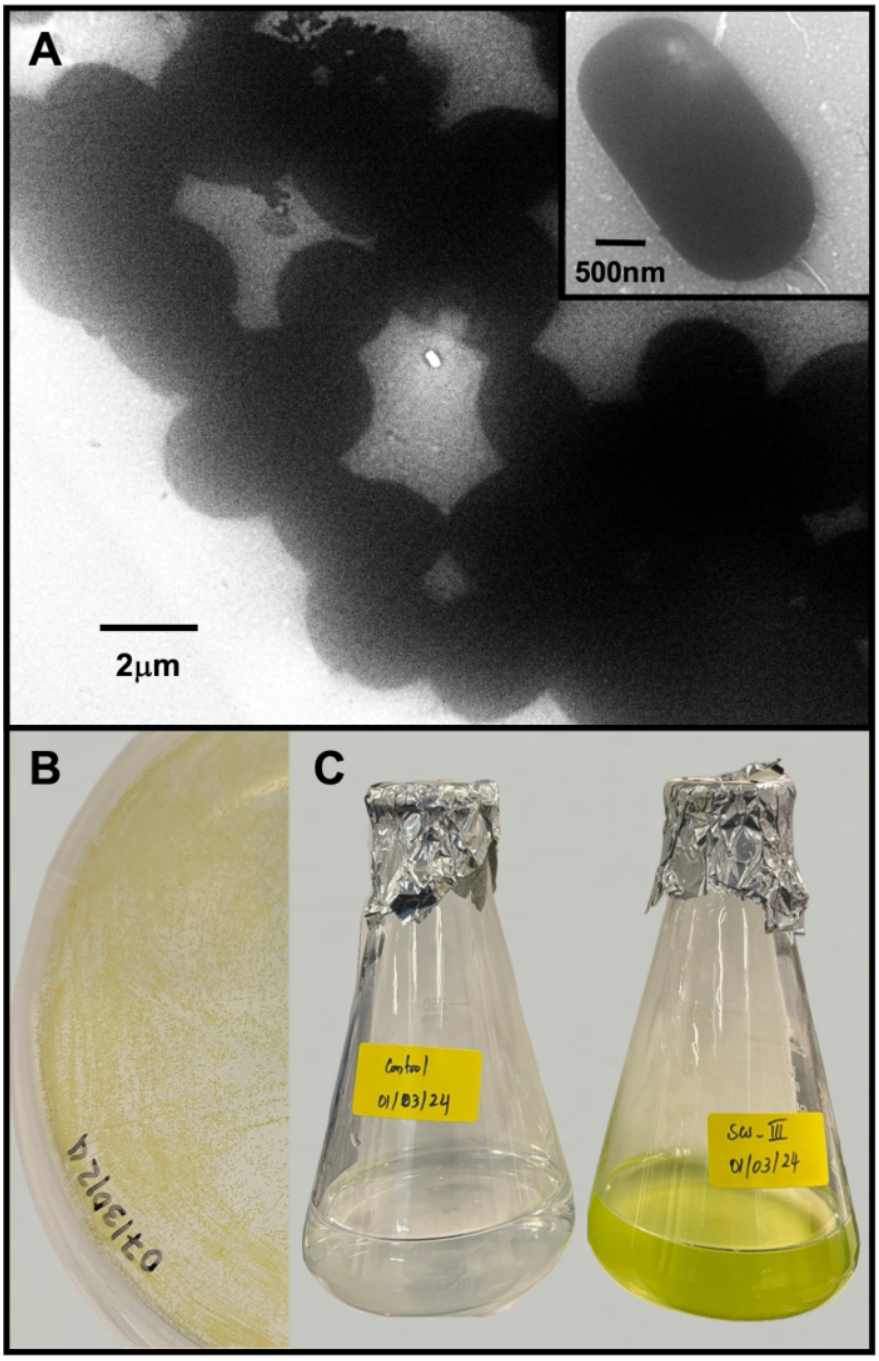

Figure 4: Transmission electron microscopy (TEM) and cultures of unknown strain SWIII. (**A**) TEM of cyanobacterium isolate designated as strain SWIII reveals oblong cells ~0.8-2.5 µm in length x ~1.0-1.5 µm in width; isolate SWIII is suspected of being a species of *Synechococcus*. (**B**) Streak plates show that strain SWII forms small yellow-green colonies

with sharp boundaries on BG-11 agar plates. (**C**) Liquid culture of SWIII reveals light green cell growth in BG-11 media.

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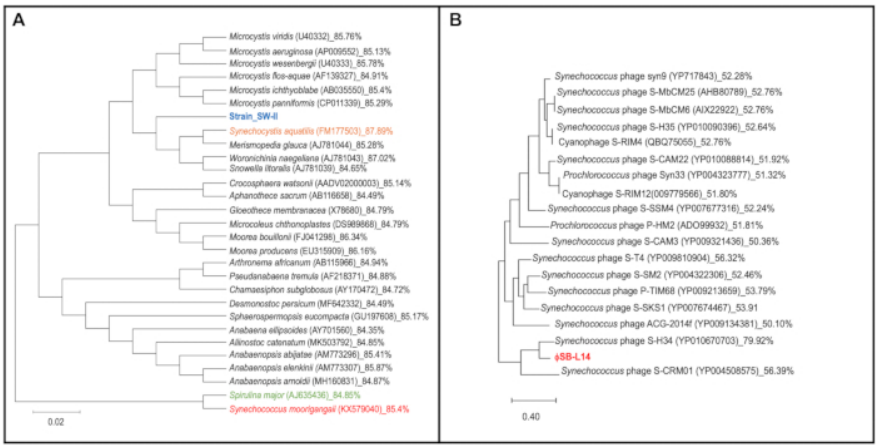

Figure 5: Phylogenies for cyanobacterial strain SWII (16S rRNA) and fSBL14 (vDNAP). (**A**) A phylogenetic analysis of cyanobacteria strain SWII was examined using the 16S rRNA gene. The resulting phylogeny suggests that SWII is likely a species of the Order Synechococcales with sequence similarity to both the genus *Synechocystis* and *Merismopedia*. Strain SWII is likely a species of the family *Merismopediaceae*, perhaps the genus *Synechocystis* based on morphology. The analysis shows 87.89% rRNA sequence similarity between sSWII and *Synechocystis aquatilis*.(**B**) Phylogenetic analysis using the viral DNA polymerase (vDNAP) gene of φSBL14 and other characterized cyanophage indicates that bacteriophage isolate φSBL14 groups with other known cyanophage. Specifically, φSBL14 is monophyletic with *Synechococcus* phage strain S-H34 exhibiting 79.92% identity based on vDNAP sequence analysis. This phylogeny, combined with evidence that it exhibits productive infection in strain SWII, suggests that φSBL14 is a cyanophage. The maximum likelihood (ML) method was used to generate both phylogenetic trees. Please click here to view a larger version of this figure.

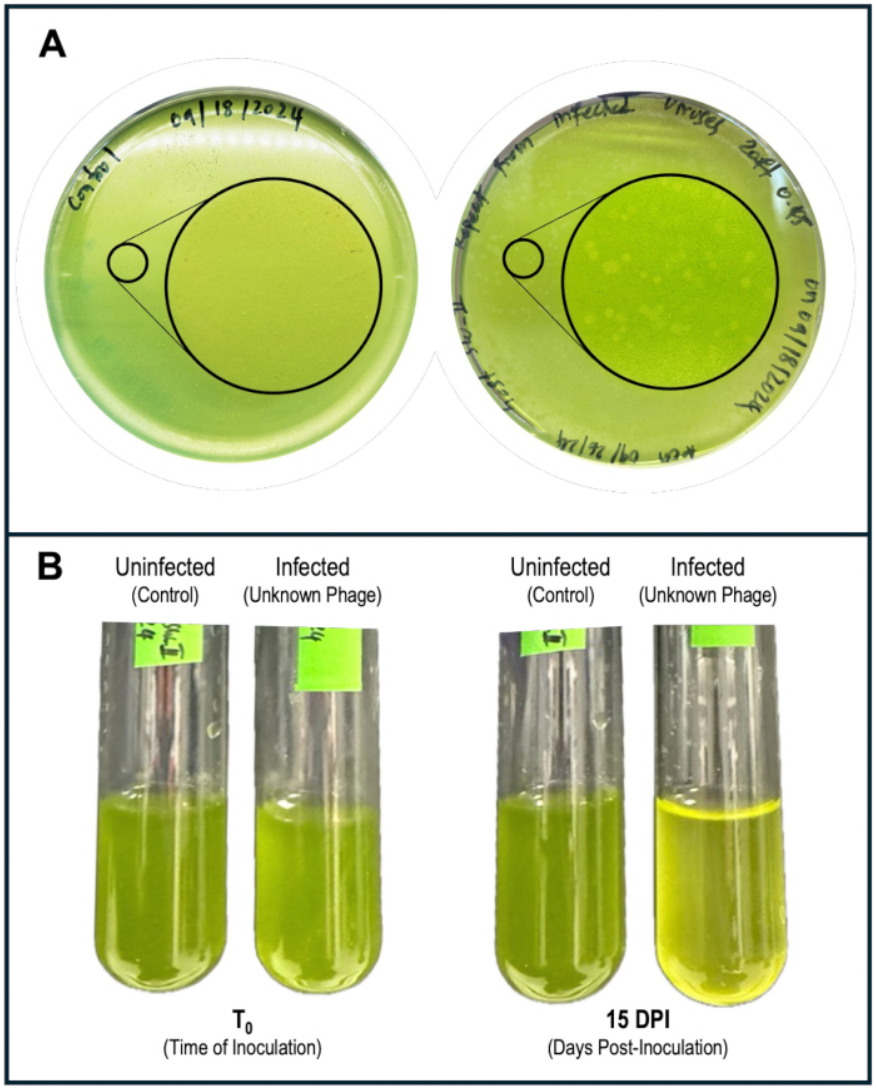

Figure 6: Strain SWII and φSBL14 infection assays on solid media plates and liquid culture. (**A**) Cyanobacteria strain SWII forms homogeneous bright green lawns on BG-11 agar plates [left]. Suspension containing cyanophage φSBL14 spread across a lawn of strain SWII results in the plaque formation at 15 days post-inoculation [right]. (**B**) Cyanobacteria strain SWII readily grows in liquid cultures of BG-11 media, producing a bright green cell suspension. Adding φSBL14 to the culture diminishes cell density and results in cell debris at the bottom of the tube and floating particulates, suggesting cell lysis due to cyanophage φSBL14 at 15 days post-infection [far right]. Please click here to view a larger version of this figure.

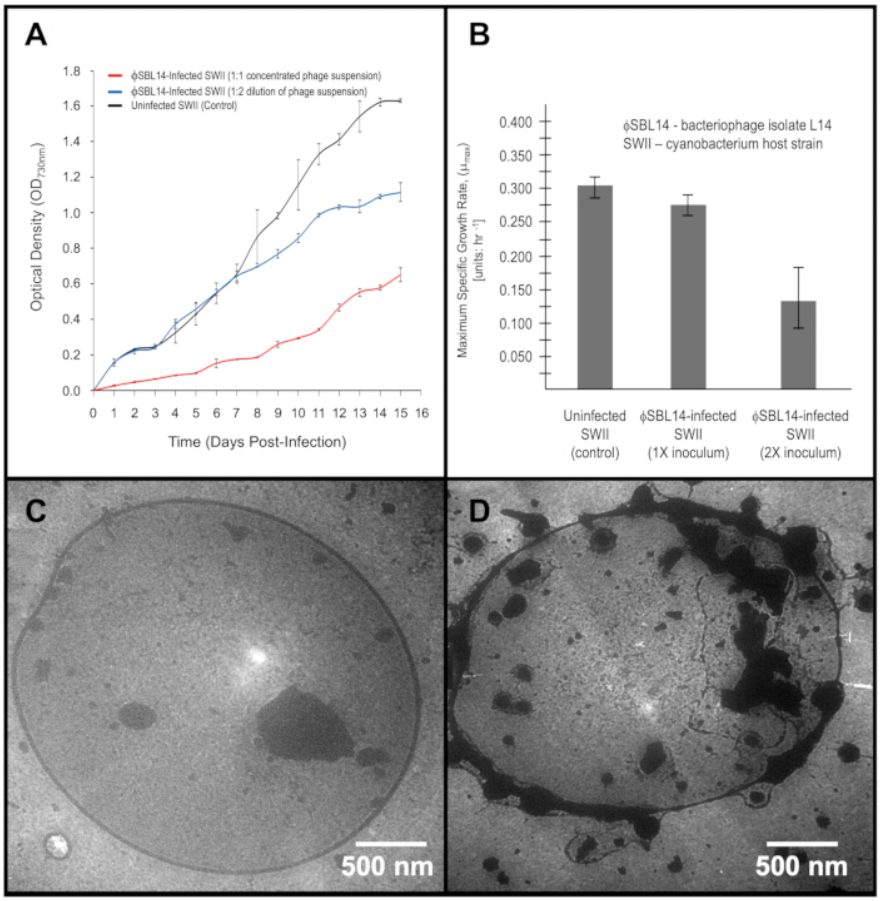

Figure 7: Qualitative and quantitative analysis of cyanobacteria strain SWII-φSBL14 infections. (**A**) Cultures of strain SWII (3 replicates per condition) are inoculated with 250 µL of 2x φSBL14 suspension and 1x φSBL14 suspension in parallel with uninfected (control) cultures and allowed to grow for 15 days. Infected growth curves exhibit retarded host growth when compared to control with the concentrated inoculum inducing a notable depression in host growth early in the growth profile compared to the diluted inoculum. Standard error (SE) bars are shown (n = 3). (**B**) Maximum specific growth rate (μ_{max}), a common metric for analyzing growth curves, shows a reduced host growth rate in cultures infected with φSBL14 when compared to uninfected control. (**C**) High-resolution transmission electron microscopy (TEM) of a cell from an uninfected control culture shows no cytopathic effects or signs of infection. (**D**) High-resolution TEM of a cell from an infected culture reveals a thickening cell membrane, irregular intracellular granules, and what appear to be blebbing from the cell membrane, all cytopathic effects typical of a bacteriophage infection. These high-resolution TEM images support the suggestion that cell death in strain SWII cultures is due to infection by φSBL14. Please click here to view a larger version of this figure.

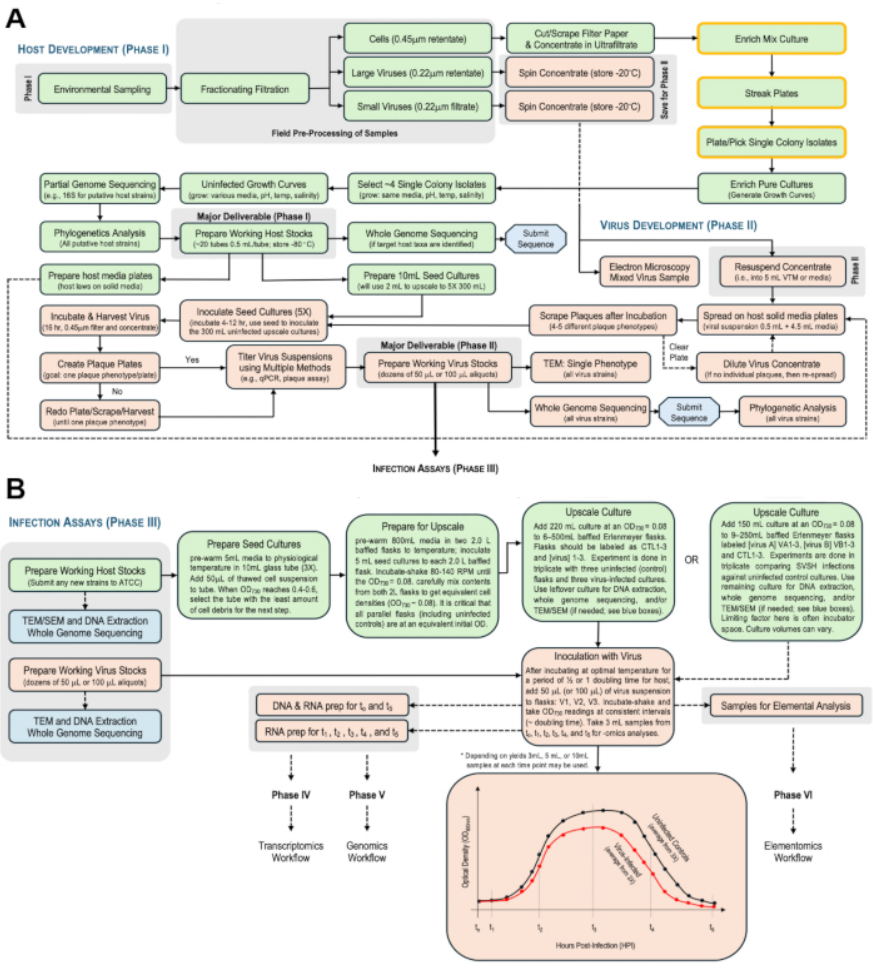

Figure 8: Comprehensive virus-host discovery and characterization workflow. (**A**) Workflow diagram showing all steps from sample collection through production of key deliverables. Steps at which the protocol may be modified to target taxa of scientific interest from mixed cultures of photosynthetic microbes (e.g., cyanobacteria versus microalgae) are designated by yellow outlines. (**B**) Workflow diagram for characterizing bacteriophage-host or virus-host interaction profiles by both solid media and liquid culture infection assays. Also shown are deliverable for advanced multi-omics workflows (not shown).

Please click here to view a larger version of this figure.

Infection Metrics	Max Growth (mmax)	Relative Resilience (RR)	Relative Virulence (VR)
Uninfected SWII (Control)	0.312	-	-
SWII - φSBL14 1X virus suspension	0.275	0.1042683	9.59063949
SWII - φSBL14 2X concentrated suspension	0.139	0.01486741	67.261211

Table 1: Calculation of Relative Virulence (VR) and Host Resilience (RR) with φSBL14 (2x concentrated phage suspension and 1x phage suspension).

Discussion

The virosphere is vast and diverse, thus requiring different experimental approaches for studying different virus systems. This includes different strategies for isolating viruses and their hosts from different environments and different processing methods to ensure that pure cultures of hosts and their viruses can be produced from raw samples. These pure cultures can be used in SVSH infection assays, both on solid media and in liquid culture, so that system features and quantitative analyses of virus-host dynamics can be achieved. In this report, we describe a standard but modifiable workflow to sample from the ocean, lakes, waters beneath ice-covered lakes, and other aquatic environments (e.g., geothermal pools and hot springs). The workflow includes details on how to pre-process samples in the field prior to shipment back to the lab, which is critical to ensure that viable microbiota make it back to the lab for more detailed analysis. Separating fractions of the raw sample by filtering and filter-concentrating through a set of filters of different sizes and protecting samples from factors such as sunlight (i.e., UV radiation) and temperature extremes are key to this first step in the workflow (**Figure 8**).

Once in the lab, virus fractions are further processed by spin-concentrating 0.45 µm filtrate directly or from biomass accumulated on a 0.22 µm filter from filtering 0.45 µm filtrate. This allows the capture of eukaryotic viruses or larger prokaryotic viruses (e.g., bacteriophage, archaeal viruses). Spin-concentrating ultrafiltrate (e.g., filtrate processed through a 0.22 µm filter) captures smaller viruses and bacteriophages. In either case, concentrated suspension is stored at 4 °C or -20 °C for future development of single-genotype bacteriophage (or virus) strains.

Once in the lab, cellular fractions from biomass accumulated on 0.45 µm filters from filtering a raw water sample are enriched using media that is appropriate (e.g., BG-11) for targeting species of the taxonomic group of interest (e.g., Synechococcales). The protocol is modifiable. By using different enrichment media, other families of microorganisms are targeted. Upon enrichment of mixed cultures, streaking solid media (e.g., agar) plates with cell suspension produces single colonies of microorganisms, each colony phenotype of which likely represents one genotype. Selecting multiple single colony isolates (SCI) with distinct colony phenotypes and inoculating seed cultures with fresh media yields pure cultures (i.e., culture comprised of one genotype). These

are the first key deliverables in the workflow. Microscopy (e.g., light microscopy) and partial genome analysis (e.g., 16S RNA) confirm the attainment of a pure culture and identify the taxonomic placement of the isolate. These purified strains serve as putative hosts for bacteriophage (or viruses) captured in the same raw sample.

To determine if any of the pure cultures are indeed hosts for bacteriophage (or viruses) from the same sample, lawns are grown on solid media plates, and dilutions of bacteriophage (or virus) suspension are spread onto the plates. Any divots, plaques, or halos in the lawn indicate a potential virus-host relationship. Scraping multiple plaques individually and inoculating individual seed cultures containing cell suspension permits propagation of a single bacteriophage (or virus) genotype, ultimately producing a pure bacteriophage suspension or virus suspension. These virus suspensions are titered using a variety of techniques, including serial dilution plaque assays (pfu/mL), qPCR (GCN_{viral}/mL), and electrospray ionization/mass spectrometry (VP/mL). Transmission electron microscopy (TEM) reveals the presence of virus-like particles (VLPs) in the culture and provides information about bacteriophage (or virus) morphology and size. Scanning electron microscopy (SEM) reveals the presence of any cytopathic effects due to virus infection of the host. DNA extraction from cell-free bacteriophage (or virus) suspensions, followed by sequencing and phylogenetic analysis, confirms the presence of the virus and taxonomic relationships. Multiple single-genotype bacteriophage (or virus) suspensions are the second workflow deliverable.

After developing a library of pure host cultures and pure bacteriophage (or virus) suspensions, baseline features of virus-host system profiles and infection dynamics are qualitatively and quantitatively described through plate-based and liquid culture infection assays. By performing single-virus/single-host (SVSH) pairings, infection assays elucidate virus replication strategy, relative virulence (V_R), relative resilience of host (R_R), growth rates, cytopathic effects, and more. The workflow presented provides a basis for more advanced characterization of bacteriophage (or virus) systems. Specifically, beyond the scope of this report, samples harvested from SVSH liquid culture infections at intermediate and end points during the infection dynamic are used to elucidate -omics substrates underlying the observed physiology. For example, transcriptome analysis (e.g., RNAseq) may be used to compare upregulation or downregulation of host or viral genes during different points along the growth curve in infected versus uninfected (control) cultures. Once the key deliverables and baseline measures have been achieved per the described workflow, more advanced questions are tractable and readily addressed.

Such questions include: What host genes or proteins are active during the eclipse phase of the viral infection cycle? What proteins are required to induce egress of progeny virions from the host? What genes endow a host with resilience to infection? How do infection dynamics change if hosts are challenged with multiple viruses in multi-virus/single-host (MVSH) infections? What are outcomes from single-virus/multi-host (SVMH) or multi-virus/multi-host (MVMH) infections? What genes drive the emergence of high-virulence bacteriophage (or virus) strains in a population? What genes drive the attenuation of a virulent bacteriophage? How does the ecological stoichiometry (e.g., element/nutrient flux and mass balance) vary between the infected and uninfected states, and how does that impact the broader ecosystem?

In summary, our workflow, which is a composite of several other protocols that target specific virus and bacteriophage systems, forms a foundation for studying almost any virus system in Bacteria, Archaea, and single-celled Eukaryotes, particularly for those hosts that are able to form homogeneous lawns or high confluency growth on plates.

Disclosures

The authors declare no conflicts of interest.

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