Supplementary Information

Williams et al.

***Halobacteriovorax*, an underestimated player in predation on bacteria: potential impact relative to viruses on bacterial mortality**

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Sampling

Ten liters of surface water was collected from both sides of a dock from the Apalachicola Bay in northwest Florida (USA) using a sterile sampler. The samples were transported on ice packs in a cooler to the Microbial Ecology Laboratory at Florida A&M University. The water collected was used to establish the microcosms described below, with all permutations, within 5 h of sampling.

Establishment of Microcosms

To create the microcosms, water samples were filtered through 0.8 µm Nuclepore track-etched membrane filters (Whatman laboratory, NJ) to remove debris and larger organisms (Fig. 1a). Aliquots were removed from filtered water samples for initial virus enumeration. Then 250 ml was dispensed into each of four 1-liter flasks to create the various microcosms, after appropriate manipulation as described below. For the experimental test microcosm, a suspension of *Vibrio parahaemolyticus* P5 was prepared by adding 5 ml of 70% artificial seawater (Instant Ocean, Aquarium Systems, Inc., Mentor, OH, USA) (pH 8, salinity 22) into an overnight culture of *V. parahaemolyticus* grown on seawater yeast extract (SWYE) agar culture plates (Kaneko and Colwell 1973) The prey suspension was spiked into the microcosm flask to yield an optical density (OD) of 0.7 at 600 nm, which corresponds to approximately 5×10^{8} CFU ml^{-1} as enumerated by CFU on SWYE Agar. An equal volume of the prey suspension was spiked into microcosms consisting of autoclaved and 0.1 µm filtered water samples that served, respectively, as the predator-free prey control and the virus-prey control. One microcosm flask did not receive any input of prey and served as the no treatment natural water (NW) control.

For the virus control microcosm, additional steps were taken to assure the exclusion of *Halobacteriovorax* from the microcosm water, as they could interfere with the results. To accomplish this, the water sample was filtered and centrifuged in a series of steps. Five hundred ml of the original 0.8 µm filtered water sample was filtered through 0.2 µm Nuclepore track-etched membrane filters (Whatman, NJ), dispensed into two autoclaved 250 ml centrifuge bottles, and centrifuged at 12,000 rpm for 30 min in an Eppendorf Centrifuge Model 5417C (Hauppauge, NY). The supernatant fluid was filtered through a disposable Nalgene® Filter Unit (Nalgene, NY, USA) with a 0.2 µm pore size, and the filtrate was centrifuged as described above. Again the supernatant fluid was filtered through a 0.2 µm pore size Nalgene® Filter Unit. In a final step, the filtrate was filtered through 0.1 um Nuclepore track-etched membrane filters (Whatman, NJ), and 250 ml of this water was dispensed into a 2-l Erlenmeyer flask to serve as the virus control microcosm.

V. parahaemolyticus strain P-5 (*V. parahaemolyticus*) was selected as prey in this study, as it is known to be susceptible to both bacteriophage (Crothers-Stomps et al 2010) and *Halobacteriovorax* found in salt water systems

All microcosm flasks were shaken at room temperature, and changes in prey density were monitored at 24 h intervals from 0 h through 120 h by OD measurements (at 600 nm) using a 48-well microtiter plate and an Absorbance Microplate Reader Q4 (BIO-TEK Instruments Inc., USA). The *Halobacteriovorax*, *V. parahaemolyticus*, and total bacteria were also monitored for population changes by qPCR as described below.

Quantitative Real-Time PCR (qPCR)

The enumeration of *Halobacteriovorax*, *V. parahaemolyticus*, and total bacterial 16S rRNA gene copies in the samples was performed with quantitative real-time PCR. Briefly, 1 ml of sample was removed from

each microcosm flask at 24-h intervals, and genomic DNA was extracted using the QIAamp DNA Mini kit (QIAGEN) at a final elution volume of 100 µl.

In the case of *Halobacteriovorax* and total bacteria, the quantification was based on the fluorescence intensity of the SYBR Green I dye, and reactions for each sample were carried out in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A *Halobacteriovorax*-specific primer set - 519F (5′-CAGCAGCCGCGGTATAC-3′) and 677R (5′-CGGATTTTACCCCTACATGC-3′), which generates an amplicon of 159 bases, was used for quantification of *Halobacteriovorax* (Zheng et al 2008). Thermal cycling conditions were: 2 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 10 s at 62 °C, and 10 s at 72 °C. The estimation of the total bacterial community was based on the quantification of the bacterial 16S rRNA gene copies using the primer pair 341F (5′-CCTACGGGAGGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGGCA-3′) (Wessén et al 2010), which generates a 194-base amplicon. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The reactions were performed in a total volume of 25 µl, which were composed of 2X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 1 µl of each primer (10 pmol μl^{-1}), 5 µl of template DNA diluted 1:10, and 5.5 µl of MiliQ water. Data were retrieved at 72 °C, and all reactions were finished with a melting curve starting at 72 °C, with an increase of 0.5 °C up to 95 °C to verify amplicon specificity (specific temperature between 85.50–86.50 °C).

The quantification of total *V. parahaemolyticus* was performed following the fluorophore-based detection of the *tlh* gene. A previously described set of primers and probe were used. The forward primer 5′- ACTCAACACAAGAAGAGATCGACAA-3′), the reverse primer 5′ GATGAGCGGTTGATGTCCAA-3′), and the probe 5′-Cy5- CGCTCGCGTTCACGAAACCGT-3′) have been previously described by Nordstrom et al. (Nordstrom et al 2007). The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 45 cycles of 5 s at 94 °C and 45 s at 59 °C. The reactions were performed in a

total volume of 25 µl, which were composed of 2X iQ Supermix (Bio-Rad, Hercules, CA, USA), 1 µl of each primer (10 pmol μl^{-1}) and 0.1 µl of fluorogenic probe (10 pmol μl^{-1}), 5 µl of DNA diluted 1:10 and 5.4 µl of MiliQ water.

Each sample was measured in triplicate, and negative controls (no template-NTC) were included. Natural water samples' template DNA was not diluted. Standard curves for each assay were obtained using serial 10-fold dilutions of linearized plasmids containing a fragment of either the *Halobacteriovorax* or the *Escherichia coli* ML-35 16S rRNA gene or an insert of the *tlh* gene of *V. parahaemolyticus*. The *Escherichia coli* ML-35 16S rRNA gene was aligned against the *V. parahaemolyticus* 16S RNA gene to confirm sequence conservation at the binding sites of the 341F-534R primers and suitability of this pair to be used in the assay. Amplification efficiency (E) was calculated using the slope of the log standard curve given by the CFX96 software: $E = 10^{(-1/slope)}$. Efficiencies ranged from 100 to 108% and averaged 100% (R^2=0.984) for the *V. parahaemolyticus* assay, 102% (R^2=0.995) for the *Halobacteriovorax* assay, and 108% (R^2=0.995) for the total bacteria assay.

Viruses and total bacteria enumeration by epifluorescence microscopy.

Samples were collected in sterile polypropylene tubes and immediately preserved with 0.02-µm filtered formalin to a final concentration of 2%. Samples were stored at 4 °C until further analysis. For virus counting, samples were stained with SYBR Gold according to Chen et al. (Chen et al 2001) and others .

Viral-like particles were counted using an epifluorescence Axioskop Trinocular Microscope (Zeiss, Thornwood, NY)**.** Total bacterial counts were conducted on formalin-preserved samples. Briefly 1 ml of the sample was stained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.01%, filtered onto 0.2µm polycarbonate filter, and counted by epifluorescence microscopy under UV excitation.

Challenge Experiment II SIP

To more specifically identify the predator involved in predation of the prey, the experiment was repeated, but with a staple-isotope-labeled *V.parahaemolyticus* prey (Challenge Experiment II SIP). To prepare the labelled prey, *V. parahaemolyticus* P-5, was grown in ISOGRO medium containing a C-13 labeled substrate (glucose). A 4-l water sample collected from the Apalachicola Bay in northwest Florida (USA) was filtered through a 0.8 μm filter, as shown in figure 2B. After aliquots were removed from the sample for determining initial total bacterial and viral counts, approximately 500 ml of the filtered water was decanted into each of five one-liter flasks. The SIP-labelled prey microcosm was established by spiking a suspension of the labeled prey into one of the flasks. An unlabeled SIP prey control microcosm consisted of unlabeled *V. parahaemolyticus* prey to allow a comparison of the typical predator-prey population dynamics to *Halobacteriovorax* predation on the labeled prey so as to detect any alterations in predation patterns resulting from the label. Other control microcosms included a *V. parahaemolyticus* prey (SIP Vp) control microcosm consisting of a prey suspension in autoclaved estuarine water, and a SIP virus control microcosm consisting of 0.1-μm filtered estuarine water with native viruses amended with the prey, but free of *Halobacteriovorax* or other bacteria removed by the filtration. Both of these controls were described above for Challenge Experiment I. In all microcosms, changes in prey density were monitored by OD. Changes in *Halobacteriovorax* and *V. parahaemolyticus* densities were also measured by plaque assay and enumeration of colony-forming units (CFU), respectively. Viruses were enumerated by direct microscopic count as described for Challenge Experiment I above.

To monitor predation by *Halobacteriovorax* by molecular methods, DNA was extracted from 100 μl of water samples from the experimental flask at 0, 48, and 120 hours and stored at –20 °C until further analysis. C^{13} DNA was separated from unlabelled DNA by density gradient centrifugation according to Padmanabhan et al. (Padmanabhan et al 2003). Briefly 100 μl of DNA was diluted in 3.9 ml of TE, to

which 4.0 grams of $CsCl_2$ was added and shaken gently. Ethidium bromide (200 µl;10 mg/ml) was added to each ultracentrifuge tube, which was then sealed and centrifuged at 50,000 rpm (251271g) for 18 hours. Labeled and unlabeled DNA separated into higher and lower bands. Centrifuge tubes were pierced with an 18-gauge needle just below the C^{13} DNA (lower heavier band), and the DNA was withdrawn. Ethidium bromide was removed from the DNA by washing several times with TE-saturated N-butanol. DNA was purified according to standard methods (Sambrook 1989). Bacterial communities were characterized by PCR-denaturing gradient gel electrophoresis (DGGE) utilizing both universal primers, GM5F (5'-CCTACGGGAGGCAGCAG-3') with a GC clamp attached to its 5' end and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') (Muyzer et al 1995), and BALO-specific primers, Bac-676F (5'-ATTTCGCATGTAGGGGTA-3') and Bac-1442R (5'-GCCACGGCTTCAGGTAAG-3') (Davidov et al 2006) by puReTaq Ready-To-Go PCR Beads (GE Healthcare Bio-Sciences) (Fig. S3). Prominent bands were excised from the gel, and DNA was eluted in 40 µl of milliQ ultra-pure water, re-amplified using the same primers without GC clamp, and sequenced. Analyses of DNA sequences were completed with the BLAST server from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Cultivation of *Halobacteriovorax* and V. *parahaemolyticus*

An aliquot of the original water sample was removed, and *Halobacteriovorax* plaque-forming units (PFU) were enumerated using the double agar overlay method with *V. parahaemolyticus* as prey, as described previously (Schoeffield and Williams 1990). For enumeration of *V. parahaemolyticus*, aliquots were plated on T1N3 (1% tryptone, 3% NaCl, and 2% agar) agar plates and specifically identified by the colony lift method (McCarthy et al 2000). Briefly, 10-fold serial dilutions of control and test samples were spread on T1N3 agar plates, and the plates were incubated at 30 °C for 16–18 hrs. Colony lifts were performed on plates with 100–300 colonies. The filters from both the samples were hybridized with alkaline-phosphatase-labeled DNA probes targeting the thermolabile hemolysin (*tlh*) for enumeration of

total *Vibrio parahaemolyticus* CFU. Total cultivable bacteria were estimated by the spread plate method onto diluted (1:20) seawater yeast extract (SWYE) agar medium (Weiner et al 1980).

Statistical Methods

The abundance of predator and prey (log transformed) were analyzed by analysis of variance (ANOVA) test followed by post hoc Holm-Sidak test to detect significant differences among the numbers of bacteria in the various microcosm treatments when Kolmogorov-Smirnov normality test were passed.

The t-test was used as appropriate to compare two groups of treatments when normality tests were passed. For all statistical analyses, $P<0.05$ was considered statistically significant (GraphPad Prism version 6.02 for Windows, GraphPad Software, La Jolla, CA, USA).

Predator-prey model

The population dynamics of the *V. parahaemolyticus* and *Halobacteriovorax* were assumed to be describable by a simple predator-prey model as follows:

$$\frac{dV_{\mathrm{p}}}{dt} = -\left(GB + M_{\mathrm{v}}\right)V_{\mathrm{p}} \qquad (1)$$

$$\frac{dB}{dt} = \left(fGV_{\mathrm{p}} - M_{\mathrm{B}}\right)B \qquad (2)$$

where V_p, and B are the numbers of *V. parahaemolyticus* and *Halobacteriovorax,* respectively, M_V and M_B are the corresponding natural mortality rates, G is the grazing rate of *Halobacteriovorax* on *V. parahaemolyticus* (i.e., the fraction of *V. parahaemolyticus* cells consumed per *Halobacteriovorax* cell per hour), and f is the number of *Halobacteriovorax* that emerge from a dead *V. parahaemolyticus* cell after its contents have been utilized by the *Halobacteriovorax* predator. After assigning initial values to V_P and B, we numerically integrated this model with a time step of one hour. We assigned a value of 3 to f based on the work of Fenton et al.(Fenton et al 2010). The value of M_V (0.0032 h^{-1}) was equated to the rate of mortality of *V. parahaemolyticus* in the control culture. The remaining parameters were chosen to give a best fit to the combined log-transformed *V. parahaemolyticus* and *Halobacteriovorax* data. In performing the numerical integration, we assumed that progeny emerged from a Bdelloplast one hour after *Halobacteriovorax* penetrated the *V. parahaemolyticus*. The criterion for best fit was that the sum of the squares of the deviations of the logarithms of the experimental numbers of *V. parahaemolyticus* and *Halobacteriovorax* from the analogous logarithms of the model results be a minimum. Best-fit values of the parameter values (Fig. S1) were as follows:

$G=4.8 \times 10^{-11}$ $cell^{-1}$ h^{-1}

$M_B = 0.072$ h^{-1}

Because the maximum size of the *V. parahaemolyticus* population was 5×10^9 cells, the implication is that each *Halobacteriovorax* cell was able to consume $(4.8 \times 10^{-11})(5 \times 10^9$ cells) = 0.24 *V. parahaemolyticus* cells h^{-1}.

An alternative approach to analysis of the data is to note that Eq. (2) implies that the rate of change of the natural logarithm of the concentration of *Halobacteriovorax* cells should be a linear function of the concentration of *V. parahaemolyticus* cells if the parameters in the model are constant. To test this hypothesis, we fit the natural logarithms of the *Halobacteriovorax* and *V. parahaemolyticus* concentrations to low-order polynomials and then plotted the estimated rate of change of the natural logarithm of the *Halobacteriovorax* concentration against the estimated concentration of *V. parahaemolyticus* cells. The results (Fig. S2) were consistent with expectations at *V. parahaemolyticus* concentrations greater than about 10^9 and extrapolated to an intercept on the ordinate close to zero, which is consistent with the low rate of mortality of *Halobacteriovorax* in the control cultures, roughly 0.026 h^{-1}. However, at lower *V. parahaemolyticus* concentrations, the slope of the relationship in figure S2 becomes much steeper and extrapolates to a value of about 0.07 h^{-1}, which is the value of M_B estimated from the least squares analysis. The explanation is that the mortality rate of the *Halobacteriovorax* is negatively correlated with the concentration of their prey at low prey concentrations because the *Halobacteriovorax* expend considerable energy searching for prey. If they do not find prey, they die. Thus dM_B/dV_p is negative at low prey concentrations, and this fact accounts for the steeper slope of the relationship in figure S2 at low *V. parahaemolyticus* concentrations. It appears that after a period of time in the absence of prey the *Halobacteriovorax* go into a resting state (control culture), and in this resting state their rate of mortality is low, roughly 0.026 h^{-1}.

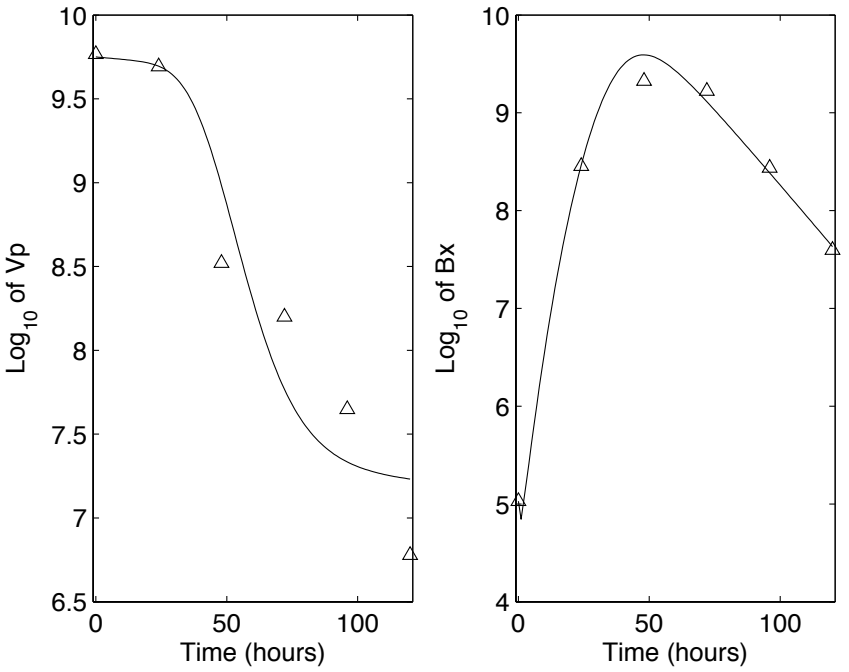

Fig.S1. Graphs of the common logarithm of numbers of *V. parahaemolyticus* (left) and *Halobacteriovorax* (right) versus time during grazing experiment. Triangles are the experimental data; the smooth curves are the model results.

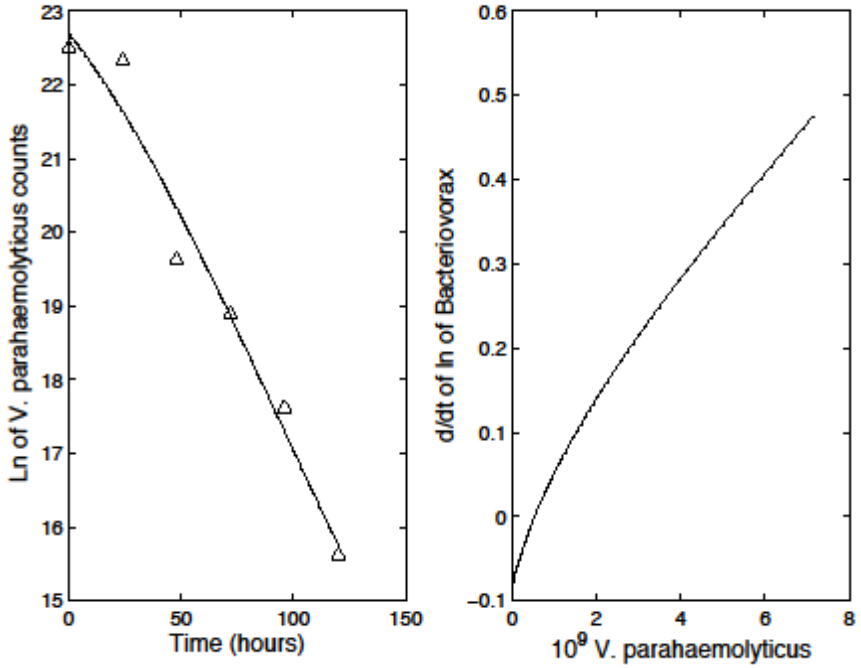

Fig. S2. Left panel: Natural logarithm of *V. parahaemolyticus* (triangles) and smooth curve of third-order polynomial fit to the data. Right panel: Rate of change of the natural logarithm of the *Halobacteriovorax* concentrations estimated from derivative of low-order polynomial fit to the natural logarithm of the *Halobacteriovorax* concentrations versus the concentration of *V. parahaemolyticus* estimated from the exponential of the smooth curve in the left panel.

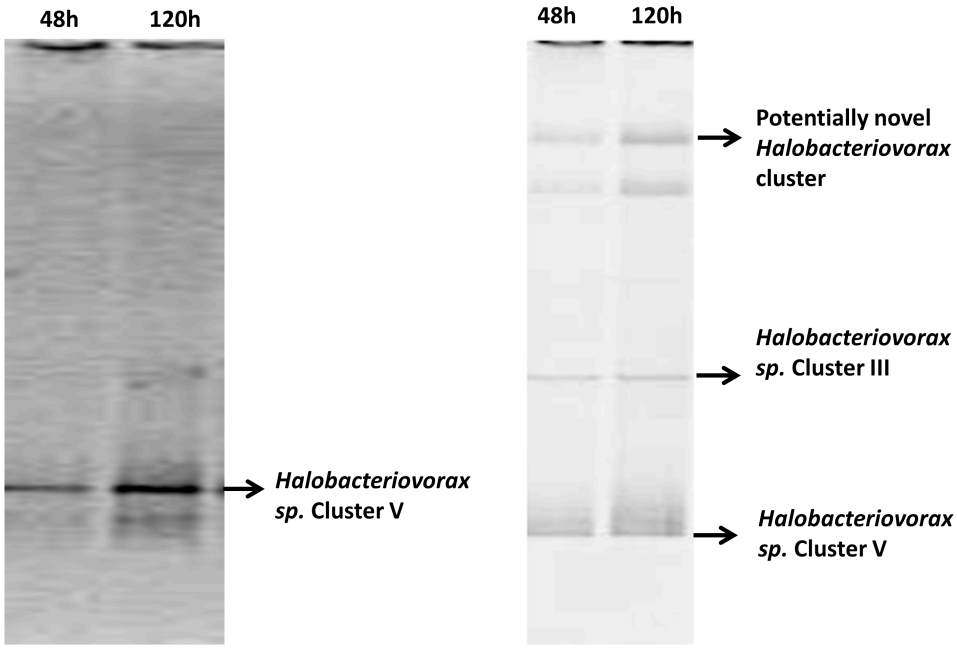

Fig. S3. DGGE profile of *Halobacteriovorax* in the 'heavier DNA' from the stable isotope probing experiment with ^{13}C labeled *V. parahaemolyticus* at 48 and 120 h. Banding pattern generated using general eubacterial primers (left) and banding pattern generated using *Halobacteriovorax* specific primers (right).

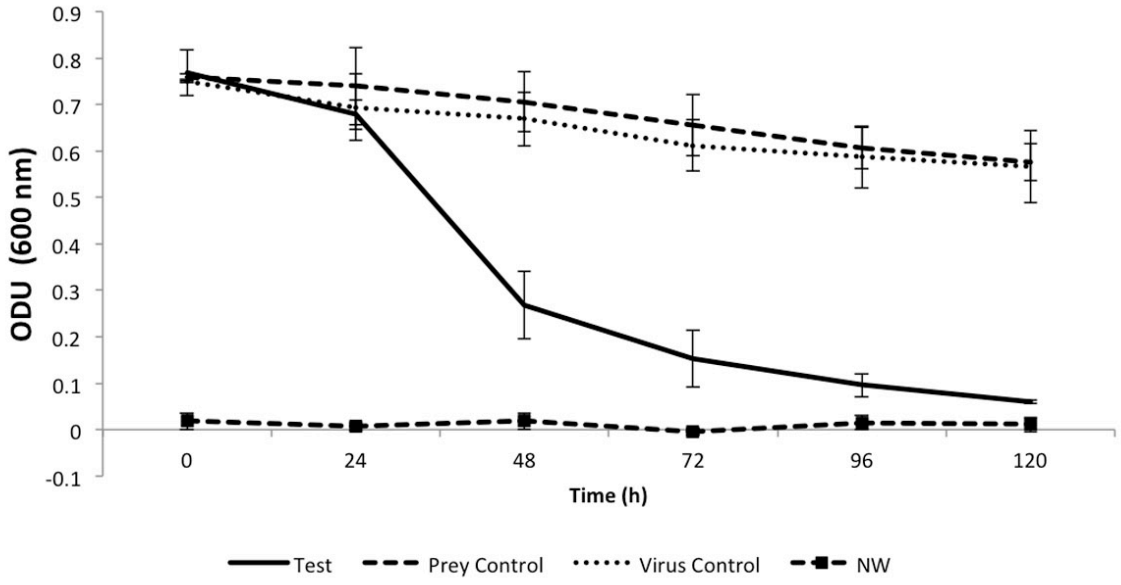

Fig. S4. Community dynamics of Challenge Experiment I as inferred by measurements of optical density units (ODU) at 600 nm.

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