



Susceptibility of two saltwater strains of *Chlorella sorokiniana* to *Vampirovibrio chlorellavorus*

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Abstract

Vampirovibrio chlorellavorus is a predatory and parasitic bacterium that thoroughly overtakes strains of *Chlorella sorokiniana* through attachment to the cell wall. Prior work has shown that many freshwater strains of *C. sorokiniana* become readily infected with this bacterium. However, saltwater strains of *C. sorokiniana* have not yet been tested for susceptibility to infection of *V. chlorellavorus*. The purpose of this study was to investigate the ability of *V. chlorellavorus* to infect two marine strains of *C. sorokiniana*: DOE 1116 and DOE 1044. These results are compared to *C. sorokiniana* DOE 1412 grown in both freshwater and saltwater environments. Laboratory-scale culture replicates of *C. sorokiniana* DOE 1412, 1116, and 1044 in different freshwater and saltwater media were infected with *V. chlorellavorus* and compared to uninfected cultures grown under the same conditions. Optical density, pulse amplitude modulation (PAM) fluorometry, and light microscopy measurements were performed to assess culture health over a 2-week period. Light and temperature remained constant throughout the course of the experiment. Microscopy results displayed clear infection of all strains of infected replicates. Further evidence for infection was provided by lower growth rates in infected cultures versus control cultures as measured by absorbance at 750 nm. Additionally, lower growth rate was observed overall for uninfected cultures of *C. sorokiniana* DOE 1412 in saltwater medium. PAM fluorometry showed slightly lower values for the maximum photosynthetic efficiency in infected cultures but the results were not statistically different than the controls. *C. sorokiniana* DOE 1412 is known to be susceptible to *V. chlorellavorus* infection in freshwater medium, BG11. Using this model system for *V. chlorellavorus* infection, our results show clear evidence of *V. chlorellavorus* infection in the two marine strains, *C. sorokiniana* 1116 and 1044.

Keywords Algal cultivation · Pathogen detection · *V. chlorellavorus* infection · Light microscopy · Host range

Introduction

Vampirovibrio chlorellavorus is a Gram-negative obligate bacterium that infects a range of freshwater green microalgae from the genus *Chlorella* (Coder and Goff 1986).

Originally named *Bdellovibrio chlorellavorus*, later reclassified as a cyanobacterium, and finally moved to the genus *Vampirovibrio*, this parasitic microorganism infects through attachment to the cell wall (Gromov and Mamkaeva 1972). *Vampirovibrio chlorellavorus* then secretes hydrolytic enzymes through a type IV secretion system to break down and ingest the contents of the algal cells. The bacterial cells are then able to replicate by binary fission on the surface of the algal cell (Soo et al. 2015). *Vampirovibrio chlorellavorus* has not been successfully cultivated outside its host (Coder and Starr 1978) making laboratory experiments challenging.

Vampirovibrio chlorellavorus has been shown to infect a number of strains of *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella kessleri*, one strain of *Chlorella saccharophila*, and one strain of *Chlorella luteoviridis* (Coder and Goff 1986). A field isolate of *C. sorokiniana* (DOE 1412), a strain with high potential as a biofuels feedstock due to its productivity in outdoor ponds and bioreactors, has demonstrated

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clear evidence of *V. chlorellavorus* infection with culture clumping, browning, and widespread death (Steichen and Brown 2019). Additionally, *V. chlorellavorus* has been observed to be a persistent algal pest in the field (Park et al. 2019). Culture clumping, browning, and overall decreased growth have been observed in outdoor algal raceways of *C. sorokiniana* DOE 1412 infected with *V. chlorellavorus* (Park et al. 2019). Both culture clumping and browning are indicative of imminent culture crash and lead to reduction in biomass values and lower productivity.

A previous study has examined the effects of varying levels of sodium chloride on the growth of *V. chlorellavorus* (Li 2015). It was determined that the addition of 0–10 g L⁻¹ of NaCl had no significant effect on the overall growth of *V. chlorellavorus* (Li 2015). To come to these conclusions, the author used the freshwater strain of *C. sorokiniana*; DOE 1412 and added salt to its medium without acclimating the strain to saltwater conditions (Li 2015). Their findings would imply that *V. chlorellavorus* can be capable of infecting strains of *Chlorella* that grow in saltwater; however, to date, the susceptibility of saltwater strains of *Chlorella sorokiniana* to *V. chlorellavorus* has not been reported. An increased understanding of the susceptibility of saltwater strains to *V. chlorellavorus* has the potential to impact algal crop protection strategies, including targeted sampling for offline molecular analysis of cultures.

Therefore, the focus of this study is to determine the susceptibility of two different saltwater strains of *Chlorella* with suitability for outdoor cultivation: *C. sorokiniana* DOE 1044, *C. sorokiniana* DOE 1116, and compare their response to *C. sorokiniana* DOE 1412 grown in traditional freshwater medium as well as acclimated to 15 ppt saltwater conditions. We find clear evidence that all strains of *C. sorokiniana* investigated are susceptible to *V. chlorellavorus* infection under the growth conditions later outlined. We provide a temporal study of the infection progression as measured with absorbance, PAM fluorometry, and brightfield microscopy.

Materials and methods

Culture maintenance

All *Chlorella sorokiniana* stock cultures were grown in an incubator at 30 °C on an orbital shaker at 150 rpm and maintained under continuous light at an intensity of 60 μmol photons m⁻² s⁻¹. Freshwater cultures of DOE 1412 were maintained in either UTEX BG-11 medium (<http://web.biosci.utexas.edu/utex/Media%20PDF/bg-11-medium.pdf>) and DISCOVER Freshwater medium (<https://discover.labworks.org/>; Supplementary Information). Saltwater cultures of DOE 1412 were acclimated through sequential inoculation of cultures in gradually higher saltwater concentrations

over time. Specifically, DISCOVER Marine 15 ppt medium (<https://discover.labworks.org/>; Supplementary Information) was added to DOE 1412 cultures growing in DISCOVER freshwater medium such that ratio of a 75% BG11 to 25% DISCOVER Marine 15 ppt was achieved and were cultivated for approximately two weeks. This cycle was repeated twice with a 50% BG11 to 50% DISCOVER Marine ratio and finally a 25% BG11 to 75% DISCOVER Marine ratio was achieved. After 2 weeks of acclimation, the cultures were placed in 100% DISCOVER Marine 15 ppt and maintained under the same conditions described above for freshwater grown cultures. Cultures of DOE 1116 and 1044 were obtained from Pacific Northwest National Laboratory growing in 35ppt DISCOVER Marine medium and maintained in that medium under the same conditions described above for freshwater grown cultures. *Vampirovibrio chlorellavorus* stocks were obtained from the Laboratory of Dr. Judy Brown at University of Arizona and grown on a benchtop orbital shaker at 24 °C under continuous lighting at an intensity of 60 μmol photons m⁻² s⁻¹ and shaken at a speed of 150 rpm. *Vampirovibrio chlorellavorus* stocks were maintained and propagated by inoculating a stock culture of *C. sorokiniana* DOE 1412 in BG11 (with OD₇₅₀ ~ 0.1) with about 20% by volume of *C. sorokiniana* culture in a mature/late-stage infection with *V. chlorellavorus*. Prior to inoculating for *V. chlorellavorus* maintenance or for an experiment, *V. chlorellavorus* stock was confirmed visually to be in the late-stage of infection at the macroscopic level by its brown color and clumping and at the microscopic level by the appearance of severely clumped, dead, hollow algal cells and high abundance of rod-shaped bacteria attached to the algae and within the medium.

Vampirovibrio chlorellavorus isolation

Vampirovibrio chlorellavorus was isolated from its host one of two ways for experiments. For experiments with DOE 1412 in DISCOVER Marine medium along with DOE 1116 and DOE 1044, 200 mL of *V. chlorellavorus* stock culture was partitioned into four 50-mL centrifuge tubes and each centrifuged at 1000 RCF for 5 min to remove the algae. The supernatant was removed and centrifuged again at 5000 RCF for 5 min to pellet the bacteria. The pellet was resuspended in 2 mL of its respective medium in each of the four tubes. These volumes were then collected in one centrifuge tube along with 12 mL of medium for a final volume of 20 mL extract solution. The extract was vortexed for 20 s before 2 mL of extract was added to inoculate each of the experimental cultures. For cultures of 1412 in BG11 and DISCOVER Freshwater medium, 20 mL of a mature/late-stage infected stock culture of *C. sorokiniana* infected with *V. chlorellavorus* was used as the inoculum. The mature/late-stage infected stock culture had been infected about 3 weeks

prior and was verified by macroscopic and microscopic appearance as described in “[Culture maintenance](#)” section.

Determining *V. chlorellavorus* concentration

Vampirovibrio chlorellavorus concentration was determined either by manual cell counting with a hemocytometer or by using an automated cell counter. For the hemocytometer method, 50 μL of *V. chlorellavorus* extract was added to a 3×1 in gridded glass slide with each individual grid square having an area of 2 mm and a thickness of 1 mm. The extract was then heat fixed and stained using a BD BBL Gram Stain Kit. The *V. chlorellavorus* was then counted manually in six squares on the grid and the average number of bacteria per square was taken. Alternatively, bacteria were counted using an automated cell counter. A 10 μL aliquot of *V. chlorellavorus* extract was inserted into a one side of a dual chamber cell counting slide (Bio-Rad, USA) and the slide was inserted into an automated cell counter (Bio-Rad TC10, USA). Replicate measurements were taken and averaged. Note: The concentrations calculated represent a total bacterial count as the gram stain and the cell counting methods are not specific for *V. chlorellavorus*. However, we believe the concentrations reported to be strongly correlated with the *V. chlorellavorus* number as visual confirmation of bacterial behavior indicates most of the bacteria are *V. chlorellavorus*.

Inoculation

For each control culture, aliquots of each stock culture were diluted to produce 50 mL cultures at an optical density of 0.1 at 750 nm. No *V. chlorellavorus* extract was added. For each infected culture of DOE 1412 in BG11 and DISCOVER Freshwater, 20 mL of mature *V. chlorellavorus* stock culture was added to produce 50 mL cultures. For each culture of DOE 1116 and 1044 in DISCOVER Marine 35ppt and DOE 1412 in DISCOVER Marine 15 ppt, 2 mL of *V. chlorellavorus* extract was added. Table 1 displays the final concentrations of *V. chlorellavorus* inoculated in the infected cultures. All flasks were placed in a shaking incubator at 30 °C under 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of continuous light and 150 rpm

Table 1 *V. chlorellavorus* inoculum concentration by strain and medium. Counts of *V. chlorellavorus* are recorded per 50 mL culture

Strain	Medium	Total <i>V. chlorellavorus</i> counts per 50 mL
1412	UTEX BG-11	2.76×10^4
1412	DISCOVER Freshwater	1.10×10^5
1412	DISCOVER Marine 15 ppt	3.73×10^5
1116	DISCOVER Marine 35 ppt	3.73×10^5
1044	DISCOVER Marine 35 ppt	3.73×10^5

for the duration of the 2-week experiment. Infected cultures and control cultures were kept in separate incubators to reduce the risk of cross contamination. Three replicates of each treatment were conducted. Additionally, all cultures were fed 10 mL of their respective medium each week.

Data collection

Over the period of 15 days, absorbance readings were taken every weekday using a plate reader (Synergy H4, Biotek Inc., USA). Then, 200 μL of culture was removed from each flask using sterile technique and placed in a 9-well plate along with its respective medium as a blank. OD measurements at 680 nm and 750 nm were taken on three replicates per experimental culture and then the average absorbance value was recorded. In addition, pulse amplitude modulation (PAM) fluorescence measurements of the effective photosynthetic efficiency (Schreiber 2004) were taken every weekday using a photosynthesis yield analyzer (Mini PAM II, Heinz Walz GmbH, Germany). Flasks were dark acclimated for a period of 10 min. After zeroing the fiber optic probe while capped, the probe was placed in near contact to the flask in an area containing the culture. Three replicate measurements were made of the maximal efficiency or quantum yield, F_v/F_m and the values averaged for each flask. Brightfield microscopy was performed approximately twice a week. Further, 500 μL aliquots from algal cultures were taken using sterile technique and placed in a 1.5 mL microcentrifuge tube. The aliquot was either centrifuged for 1 min at ~650 rpm or left sitting in a tube stand for 5 min to concentrate the cells slightly. A 10 μL aliquot from the concentrated sample and placed onto a standard microscope slide. A coverslip (#1.5) was applied and sealed using nail polish. The slide was placed on the stage of an inverted microscope (IX71, Olympus, Japan) equipped with a halogen lamp (TH4-100, Olympus, Japan) and a three-color CCD (Infinity2-3, Teledyne Lumenera, Canada) and a 60 \times oil objective (PLAPON60XO, Olympus, Japan). Images were acquired using Lumenera Infinity Analyze software (Version 6.5, Teledyne Lumenera, Canada). White correction balance was applied to the images in the acquisition software. Light and temperature readings were recorded weekly to verify consistency throughout the experimental period.

Results and discussion

We conducted laboratory studies to determine the susceptibility of two different saltwater strains of *Chlorella* with suitability for outdoor cultivation: *C. sorokiniana* DOE 1044, *C. sorokiniana* DOE 1116, and compared their response to *C. sorokiniana* DOE 1412 grown in BG-11, the accepted benchmark laboratory system for *V. chlorellavorus* infection.

Additionally, we investigated *V. chlorellavorus* infection *C. sorokiniana* DOE 1412 acclimated to 15ppt saltwater conditions.

To characterize the effects of *V. chlorellavorus* infection in *C. sorokiniana* cells, we employed three bulk/ensemble methods of observation (visual color change, absorbance at 750 nm, and PAM) and one single cell method of observation (brightfield microscopy). We found single cell analysis to be capable of visualizing the bacterium entering the algal cell and subsequent cellular degradation.

As described in the methods section, we utilized two different approaches for inoculating cultures with *V. chlorellavorus*: an aliquot of mature/late-stage infected culture and an aliquot of *V. chlorellavorus* isolate. Traditionally, an aliquot of mature/late stage infected culture is utilized because of the challenges associated with isolating a small, obligate bacterium from its host. However, using an aliquot of mature/late-stage infected culture leads to difficulty

quantifying bacterial concentrations as well as a high degree of variability between aliquots based on the culture maturity and conditions. Cultures inoculated with an aliquot of mature/late-stage infected cultures receive a moderate and variable number of *Chlorella* cells in various stages of infection in addition to the *V. chlorellavorus* bacteria. This leads to changes in macroscopic and microscopic appearance and absorbance measurements in the initial stages of infection that may be confounded with later stage infections. An example of this is seen in the O.D. 750 nm measurement on day 1 for the *C. sorokiniana* DOE 1412 in BG-11 infected with *V. chlorellavorus*, shown in the left column of Fig. 1. The O.D. 750 appears significantly lower for the infected culture, even though the *C. sorokiniana* DOE 1412 concentration was equal to the control prior to the addition of the inoculum. Additionally, Supplementary Fig. 1 shows an infected culture of *C. sorokiniana* DOE 1412 on day 1. The cells in this image show the three hallmark characteristics

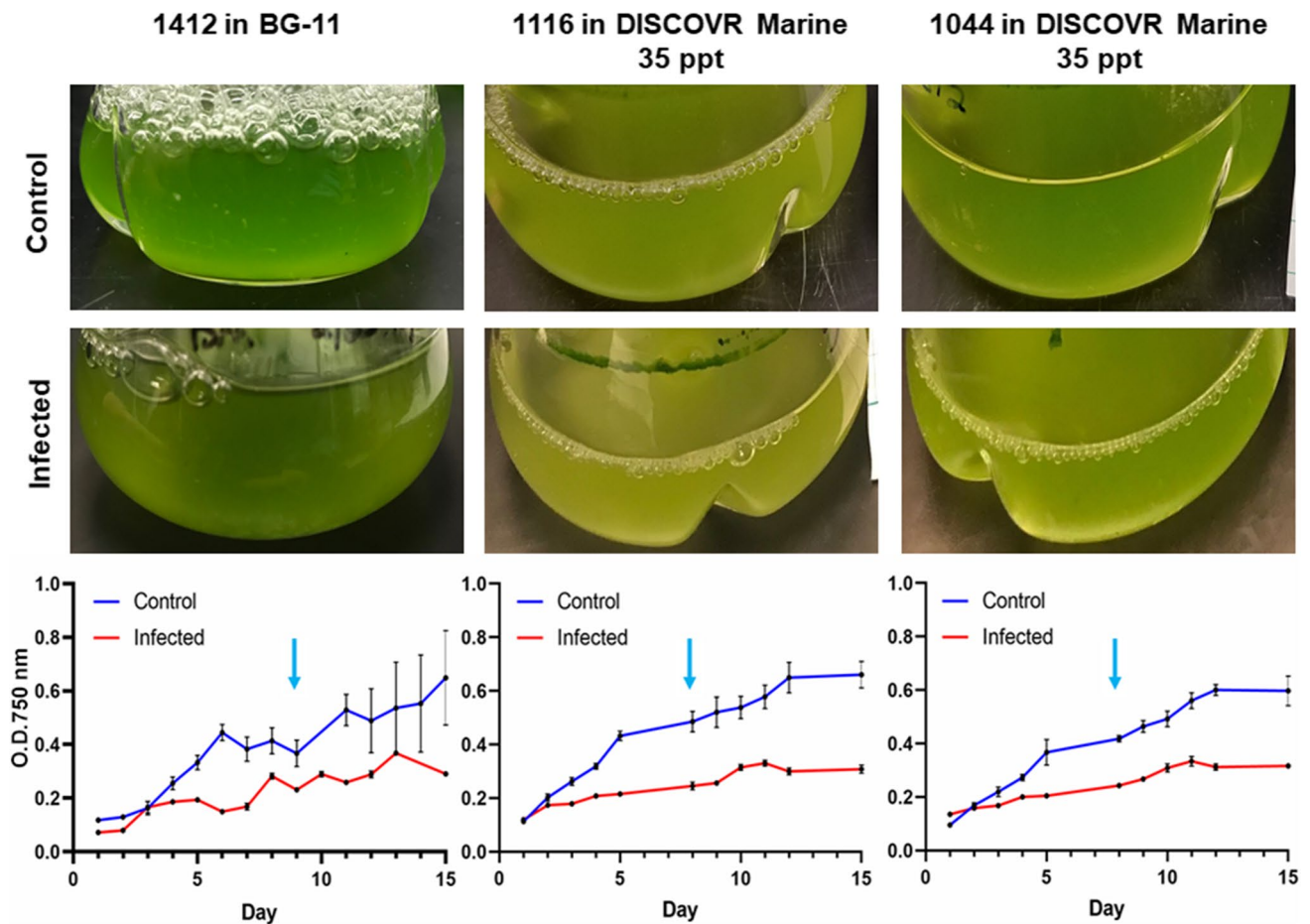


Fig. 1 Rows 1 and 2: Visual comparison of control and infected cultures of *C. sorokiniana* DOE 1412 in BG-11, DOE 1116 in DISCOVER Marine 35 ppt, and DOE 1044 in DISCOVER Marine 35ppt on day 15 of measurements. Control cultures are displayed on the top row and infected cultures are shown on the bottom row. Row 3:

15-day absorbance results at 750 nm of *C. sorokiniana* DOE 1412 in BG-11, DOE 1116 in DISCOVER Marine 35 ppt, and DOE 1044 in DISCOVER Marine 35 ppt. Turquoise arrows indicate culture feedings. Data points represent the average of 3 biological replicates. Error bars display standard deviation

Table 2 Average change in absorbance at 750 nm of control and infected cultures from day 1 to day 15. Values were averaged from three replicate measurements and the standard deviation of each strain and medium combination is reported

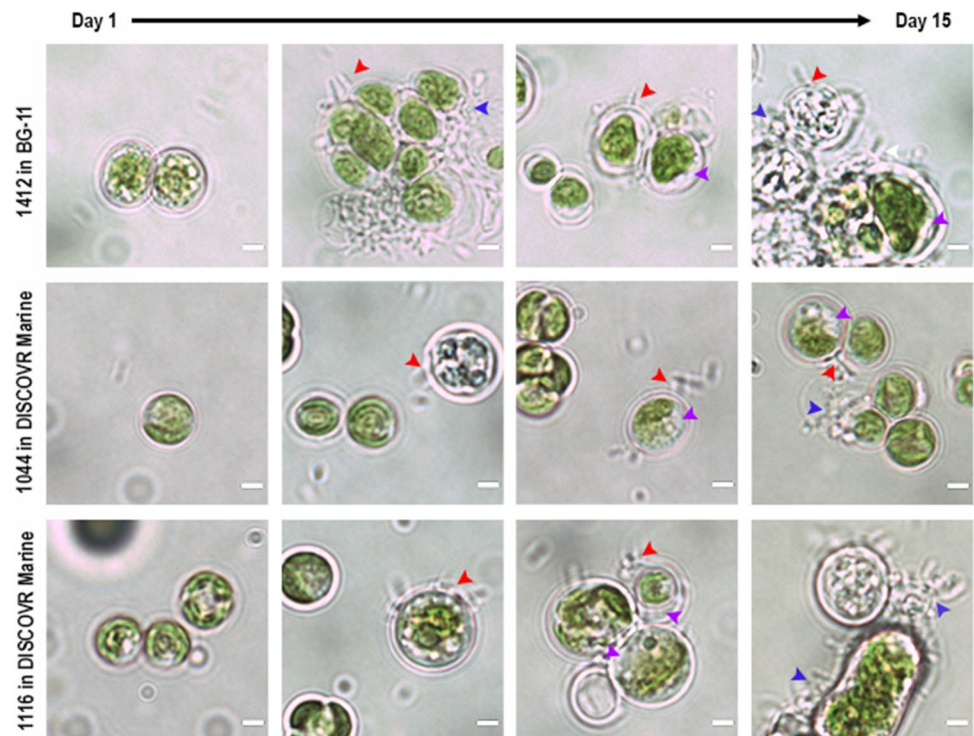
Strain	Medium	Control		Infected	
		Average absorbance change	Standard deviation	Average absorbance change	Standard deviation
1412	UTEX BG-11	0.53	0.17	0.22	0.01
1412	DISCOVER Freshwater	0.81	0.05	0.37	0.05
1412	DISCOVER Marine 15 ppt	0.39	0.07	0.07	0.01
1116	DISCOVER Marine 35 ppt	0.55	0.04	0.19	0.02
1044	DISCOVER Marine 35 ppt	0.50	0.05	0.18	0.01

of a mature/late-stage infection—(1) strong clumping; (2) clear, hollow cells without chlorophyll; and (3) multiple rod-shaped bacteria attached to and within the cells. To avoid misinterpretations and inconsistencies resulting from the addition of significant volumes of late stage infected algal cells, we developed an alternative method using an initial gentle centrifugation to separate the algae and a second stronger centrifugation to pellet the bacteria. It should be noted that this method was found to be relatively low yield in terms of the total number of bacteria isolated from a mature culture; the bacteria isolated are concentrated into a smaller volume permitting higher inoculation densities. The ability to concentrate pellet into a known volume ensure repeatable inoculation concentrations. An additional advantage of isolating *V. chlorellavorus* is seen when performing microscopy measurements, namely the lack of late-stage infected host algae in early stage infected cultures, thus giving a more accurate level of the infection.

From visual observations, infected cultures of DOE 1412 in BG-11, 1116, and 1044 appeared to be lighter and more yellow in color compared to the control cultures. This clear indicator of culture stress is displayed in the upper two rows of Fig. 1 with the visual comparison of control and infected cultures on day 15 of observation. It is important to note that DOE 1116 and 1044 cultures are lighter in color than DOE 1412 and that we did not compare color change between strains, only between infected and control cultures of the same strain.

Figure 1, lower row displays absorbance values at 750 nm over the time course of the experiment for control and infected cultures of DOE 1412 in BG-11, DOE 1116 in DISCOVER 35 ppt, and DOE 1044 in DISCOVER 35 ppt. Absorbance values for control and infected cultures of DOE 1412 in DISCOVER Freshwater and DISCOVER Marine 15 ppt can be found in Supplementary Fig. 2. Absorbance differences are quantified in Table 2.

Fig. 2 Bright-field microscopy images of the 15-day progression of *C. sorokiniana* DOE 1412 in BG-11, DOE 1116 in DISCOVER Marine 35 ppt, and DOE 1044 in DISCOVER Marine 35 ppt. Red arrowheads indicate *V. chlorellavorus* attachment to the algal cell. Blue arrowheads indicate multiple *V. chlorellavorus* cell attachments following replication. Purple arrowheads indicate retraction of chloroplast. Areas of interest are highlighted by magnification. Scale bar indicates 2 μm . Image saturation and RGB levels have been adjusted for improved display. All individual images were adjusted to the same values



On average, the infected cultures exhibited approximately 50% reduction of the growth rate over the course of the experiment as compared to their uninfected counterparts. The lower absorbance change in control cultures of *C. sorokiniana* DOE 1412 in DISCOVER Marine 15 ppt as compared to the same organism grown in BG-11 can be attributed to the fact that this strain was grown in saltwater conditions, which is not its ideal environment.

Microscopy images displayed clear signs of *V. chlorellavorus* infection in all infected cultures. In Fig. 2, the progression of infection from *V. chlorellavorus* attachment to the algal cell wall, replication, and widespread cell death is shown across the freshwater and saltwater strains of *C. sorokiniana* and these stages of infection are indicated with various colored arrowheads. A detailed figure panel showing additional representative images for each condition and each medium throughout the time course is included in Supplementary Figs. 4–8.

Pulse-amplitude modulation (PAM) fluorescence measurements were recorded for all strains and conditions on cultures that had been briefly dark-adapted. The PAM methodology as employed measures the maximum photochemical efficiency or quantum yield in the cultures and it was anticipated that this parameter would be sensitive to a reduction in photochemical efficiency caused by *V. chlorellavorus* infection (Schreiber et al. 1995). Interestingly, all treatments and strains of *C. sorokiniana* tested had similar values for the maximum photochemical efficiency throughout the 15-day experiment. The infected cultures showed slightly lower values than the control cultures, but these were not statistically significant. The PAM data is shown in Supplementary Fig. 3. This finding indicates that even cultures with a high degree of *V. chlorellavorus* infection could perform photosynthesis to some degree. Presumably, this is because there are still a sufficient number of cells that are not infected or in early stages of infection and underscores the need to utilize a microscopy technique with single cell resolution to identify *V. chlorellavorus* susceptibility.

Conclusion

Our results have demonstrated clear *V. chlorellavorus* infection in both *C. sorokiniana* DOE 1044 and DOE 1116, two promising marine strains for outdoor algal cultivation. Infection was demonstrated through multiple analytical techniques, with bright-field microscopy giving the unique perspective at the level of individual cells. The infection in saltwater strains DOE 1116 and 1044 exhibits similar behavior as the accepted model infection *C. sorokiniana* DOE 1412 growing in BG-11 medium. Additionally, DOE 1412 grown in DISCOVER Marine 15ppt medium

was susceptible to infection, albeit at a slightly slower progression than when grown in freshwater medium.

We show it is advantageous to evaluate infection at the single cell level with brightfield microscopy, as laboratory-scale measurements did not give definitive evidence of *V. chlorellavorus* infection, but rather generalized culture stress (e.g., lightening and yellowing of cultures, reduced growth). This study has further broadened the host range of *V. chlorellavorus* and show definitively that it can infect saltwater strains of *C. sorokiniana* to a similar extent as freshwater strains. This knowledge assists the algal grower in deciding on appropriate sampling/surveillance strategies to screen for *V. chlorellavorus* infection in saltwater strains of *C. sorokiniana*. Currently, *V. chlorellavorus* infection is potentially identified in the field based on culture color and clumping and confirmed in the laboratory with offline molecular methods (PCR, sequencing). These techniques are time-consuming and costly; therefore, they are not used for routine pest surveillance. Microscopy is often used for routine surveillance of pests, but has been limited to larger-scale, easy to identify pests such as diatoms, ciliates, and amoeba. The positive identification of *V. chlorellavorus* infection from brightfield microscopy images could be a future area of development enabling early detection of algal pests.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10811-021-02602-0>.

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Author contribution LCA: acquisition of data, methodology, experimental design, analysis and interpretation, writing-original draft, writing-review & editing. DM: experimental design, writing-review and editing, supervision. TH: acquisition of data, analysis and interpretation, writing-review and editing. JAT: project administration, conceptualization, experimental design, writing-review and editing, resources, validation, supervision, funding acquisition.

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Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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