



# Association of *Vampirovibrio chlorellavorus* with decline and death of *Chlorella sorokiniana* in outdoor reactors

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## Abstract

The outdoor ARID raceway was established for optimizing the cultivation of microalgae for biofuel production. During the summers of 2014 and 2015, discoloration was observed in cultures of *Chlorella sorokiniana* (DOE1412), which shifted from a vibrant green color to yellow, followed by cell clumping, decline in density, and rapid death, resulting in 40–60% reduced biomass production. Total DNA was purified from the raceway samples and subjected to polymerase chain reaction (PCR) amplification using degenerate primers that amplify the 16S rRNA gene of eubacteria. BLASTn analysis of the cloned amplicon sequences revealed the presence of the Gram-negative, predatory bacterium, *Vampirovibrio chlorellavorus*. Scanning electron microscopic examination showed an abundance of coccoid cells, 0.3–0.6 µm in diameter, some of which were attached to *C. sorokiniana* cells. PCR amplification indicated the presence of *V. chlorellavorus* in raceway vessels, water lines, connective tubing, and in early, scaled-up DOE1412 cultures used to inoculate the raceway. Based on PCR detection, the decontamination of the equipment and water line with “Wal-Clean” more effectively eliminated *V. chlorellavorus* and delayed the onset of attack, compared to the chlorine disinfectant, trichloromelamine (TCM). Total DNA was isolated from soil samples collected monthly from the nearby Rillito River during 2014–2015 and subjected to PCR amplification using primers designed to amplify the 16S rRNA and 18S rRNA gene of *V. chlorellavorus* and *C. sorokiniana*, respectively. Results indicated that *V. chlorellavorus* and *Chlorella* spp. were present in most of the riverbed samples nearly year round, suggesting a possible naturally occurring reservoir of the predatory bacterium.

**Keywords** Chlorophyta · Cyanobacteria · DOE1412 · Molecular detection · Polymerase chain reaction

## Introduction

During the past several decades, commercial culturing systems for microalgae have been tested for biofuel production (Sheehan et al. 1998). Modeling of key factors including temperature, sunlight, and land availability indicated that southern Arizona has one of the greatest potentials for algal biofuel

yield in the USA (Wigmosta et al. 2011). A field isolate of the green microalga *Chlorella sorokiniana* (DOE1412) was selected for cultivation in southern Arizona based on the high growth rate of 0.53 day<sup>-1</sup> under heterotrophic condition (Kim et al. 2013) and high lipid accumulation at temperatures of 20–45 °C (Venteris et al. 2014), which are common in the Sonoran Desert. The University of Arizona (UAZ) Algae Raceway Integrated Design (ARID) outdoor reactor (+32°16'49.29", -110°56'9.82") was designed to cultivate microalgae in an arid climate, and to maintain desired water temperatures between 15 and 30 °C, facilitating the optimal growth of many algal species (Waller et al. 2012). Challenges to the successful outdoor cultivation of algae involve continuous exposure to environmental stresses such as fluctuating temperatures, pH change, and storm systems and precipitation that introduce biotic contaminants, including competing algal species, and bacterial pathogens, and predators.

During the summer of 2012, the project strain of *C. sorokiniana*, DOE1412, grown in the ARID reactor, experienced an unexpected decline followed by rapid death. Upon

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close inspection, there was no evidence of predators or other obvious invaders, although the dead DOE1412 cells were devoid of chlorophyll and shriveled in appearance. The DOE1412 cells were intact, potentially ruling out viral pathogens that might be expected to cause algal cell lysis. To identify the suspected bacterial cause of death, molecular analysis of water samples containing DOE1412 was carried out by polymerase chain reaction (PCR)-amplification using degenerate primers to amplify the bacterial 16S ribosomal RNA gene (16S rRNA), followed by sequencing of cloned amplicons. The BLASTn search of the GenBank database indicated detection of the bacterium, *Vampirovibrio chlorellavorus* (Gromov and Mamkaeva 1972, 1980), which has a Gram-negative pleomorphic cocci shape and is ~0.3–0.6 µm in size and classified in the phylum Cyanobacteria (Soo et al. 2015). The predatory bacterium is an obligate pathogen of certain freshwater green algae and has been previously identified from diverse environmental samples, including freshwater sources (Gromov and Mamkaeva 1972), soil (Shi et al. 2015), bovine rumen (Das et al. 2014), and most recently, from an open pond, commercial algal (*Chlorella* spp.) production facility in the southwestern USA (Ganuza et al. 2016).

Although most features of *V. chlorellavorus* are consistent with those of other cyanobacteria, it lacks photosynthetic machinery, having evolved a predatory lifestyle as an obligate pathogen of green algae and, thus far, it has been associated with species of the green algal genus, *Chlorella*. During the infection cycle, *V. chlorellavorus* attaches to the surface of the algal cell wall and the bacterial plasmid DNA together with hydrolytic enzymes are thought to be injected into the prey cell using a type IV secretion apparatus. The bacteria and plasmids undergo binary fission and replication, respectively, and following the breakdown of cellular constituents, it is thought that they are released into the environment where nutrients are utilized by resident *V. chlorellavorus*. At a macroscopic level, this results in a rapid change in the appearance and color of the *Chlorella* host, from vibrant green, to yellow, and then brown, with clumping of algal host cells, followed by death (Soo et al. 2015).

Since its description in 1972, few studies have been conducted to elucidate the details of the life cycle of the bacterium. Initially, this predatory bacterium was given the name *Bdellovibrio chlorellavorus* and placed in the class, Deltaproteobacteria. In 1980, it was reclassified as a cyanobacterium and assigned to the genus *Vampirovibrio*, based on its distinct mode of predation. Unlike *Bdellovibrio*, which characteristically invade the periplasm of their Gram-negative bacterial hosts, *V. chlorellavorus* attaches to the surface of *C. vulgaris* (Gromov and Mamkaeva 1980; Coder and Goff 1986). A recent complete genome sequence of the *V. chlorellavorus* from 36-year-old lyophilized culture confirmed that the predatory bacterium is a member of a recently described basal lineage of non-photosynthetic Cyanobacteria,

class Melainobacteria. Annotation of the genome has shown that *V. chlorellavorus* encodes an *Agrobacterium tumefaciens*-like conjugative type IV secretion system (SS), which is hypothesized to be involved in host invasion, following attachment of the bacterium to the algal cell (Soo et al., 2015). Metabolic predictions based on gene annotations of the *V. chlorellavorus* genome revealed that this unusual cyanobacterium lacks photosynthetic or other possible carbon-fixing mechanisms, and hence its requirement for a photosynthesizing host (Soo et al. 2015). It also appears to lack a complete set of genes for the tricarboxylic acid (TCA) cycle, which are essential for energy production to maintain a predatory life cycle. However, the genome encodes genes with a predicted involvement in the synthesis of nucleotides and most essential amino acids, and those associated with the non-mevalonate pathway. In addition, the *V. chlorellavorus* genome encodes predicted genes involved in cell wall degradation, including > 100 proteases and peptidases, which are presumed to be involved in hydrolysis of the cell wall and/or cellular contents. Based on the genome annotation, a predatory life cycle model has been proposed, consisting of the following stages: (1) surveillance to locate the algal host by a vibrioid-shaped, flagellated cell, possibly using chemotaxis; (2) attachment to the algal host surface (Guglielmini et al. 2013), formation of a conjugative apparatus (such as a bacterial secretion system), followed by the injection of hydrolytic enzymes into the host cell; (3) ingestion of algal lysates to facilitate bacterial binary fission and plasmid replication; and (4) release of bacterial offspring from algal host cells (Soo et al. 2015).

A number of strategies have been tested to control losses incurred to algal production systems following attack by this predatory bacterium. Among the approaches that have been used successfully to minimize *Chlorella* spp. infection by *V. chlorellavorus* are early, intensive treatments with various chemical agents and physical and biological methods when the bacterium is present but in low numbers (Carney and Lane 2014). A recent study has shown that a pH-shock treatment in which the pH of the culture is shifted from 7.5 to 3.5, followed by a re-adjustment to pH 7.5, effectively decreased the population size of *V. chlorellavorus* without compromising algal growth. This treatment was reported significantly to increase the longevity of the algal culture and produce harvestable biomass (Ganuza et al. 2016). Bioactive metabolites produced under iron-depleted conditions also have been shown to provide protection against attack of *Chlorella* spp. by *V. chlorellavorus* (Bagwell et al. 2016).

In this study, the presence and observed detrimental effects of *V. chlorellavorus* infection on the ability to cultivate *C. sorokiniana* DOE1412 is reported following initial and repeated outbreaks in experimental outdoor reactors designed for microalgae cultivation in the arid southwestern USA. In the laboratory, methods were developed to continuously co-

culture the predatory bacterium and its *C. sorokiniana* host by the periodic serial transfer of infected cells into new media to promote growth under optimized conditions of temperature and light. Finally, a PCR assay was designed and optimized based on amplification of the 16S ribosomal RNA (rRNA) gene, to facilitate *V. chlorellavorus* detection in laboratory cultures. The molecular assay was further used to investigate the possibility that suspect algal hosts of *V. chlorellavorus* and the predatory bacterium itself could reside in the nearby riverbed, perhaps owing to its percolation in water emptied into the soil surrounding the raceway facility, and/or due to naturally occurring populations, which could serve as a source of bacterial inoculum. Here, the association of *V. chlorellavorus* with decline and death of *C. sorokiniana* in ARID outdoor reactors was established using a semi-quantitative molecular assay to monitor the presence of *V. chlorellavorus* in the ARID raceway and in nearby riverbed soil samples to understand better the natural occurrence and the potential cycling of *V. chlorellavorus* between a natural habitat and outdoor algal raceway in southern Arizona.

## Materials and methods

### DOE1412 cultivation

The *Chlorella sorokiniana* DOE1412 culture was obtained from Dr. Juergen Polle (City University of New York, Brooklyn; USA) and was among the isolates collected and characterized in the National Alliance for Advanced Biofuels and Bioproducts (NAABB), funded by the US Department of Energy (Neofotis et al. 2016).

The algal culture was maintained in the laboratory on BG-11 medium at 24 °C for a dark-light (16 h: 8 h) cycle at 108–135 PPF ( $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The medium contained 17.6 mM NaNO<sub>3</sub>, 0.22 mM K<sub>2</sub>HPO<sub>4</sub>, 0.03 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.03 mM citric acid·H<sub>2</sub>O, 0.02 mM ammonium ferric citrate, 0.002 mM Na<sub>2</sub>EDTA·2H<sub>2</sub>O, and 0.18 mM Na<sub>2</sub>CO<sub>3</sub>. Trace metals were added to the BG-11 medium to achieve a final concentration of 46  $\mu\text{M}$  H<sub>3</sub>BO<sub>3</sub>, 9  $\mu\text{M}$  MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.77  $\mu\text{M}$  ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.6  $\mu\text{M}$  Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.3  $\mu\text{M}$  CuSO<sub>4</sub>·5H<sub>2</sub>O, and 0.17  $\mu\text{M}$  Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O. For agar plates, 1 mM of sodium thiosulfate pentahydrate and 30 g agar were added to the medium. For outdoor cultivation, algal cultures were grown in 1- or 2-L flasks containing BG11 medium and transferred in sequential scale up to a 19- and then 946-L cube containers, respectively, prior to cultivation in the ARID raceway in Pecos medium. The Pecos medium contained a final concentration of 1.7 mM urea (NH<sub>2</sub>)<sub>2</sub>CO, 0.05 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1.4 mM KCl, 0.03 mM FeCl, and BG-11 trace metal solution, as described above. The ARID raceway design

and general operational information were as described by Waller et al. (2012).

### Total DNA isolation

Total DNA was isolated from the microalgal cultures including laboratory culture, raceway water sample, vessels, water lines, connective tubing, and riverbed soils using the CTAB method (Doyle and Doyle 1987) with slight modification. Briefly, the microalgal cells (5–10 mg) were collected from a 50-mL volume of algal cultures and 3 g of soil samples by microcentrifugation at 8000 rpm in an Eppendorf Centrifuge 5804 R (Brinkmann Instruments Inc., USA) for 5 min, and the supernatant was discarded. One-milliliter prewarmed CTAB buffer, containing 20  $\mu\text{L}$   $\beta$ -mercaptoethanol and 20 mg 1.4 mm metal beads (Qiagen Inc., USA), was added to each tube. The contents were pulverized for 5 min using a bead beater (Biospec Products Inc., USA). The supernatant was transferred to a 1.5-mL microfuge tube and 700  $\mu\text{L}$  chloroform/isoamyl alcohol (24:1) was added, followed by vigorous mixing. The emulsion was broken by centrifugation at 9000 rpm for 10 min. The supernatant was collected, and total nucleic acids were precipitated by the addition of 2/3 volume cold isopropanol and incubation for 2 h at –20 °C. After centrifuging at 9000 rpm for 10 min at 4 °C, the pellet was washed twice with 1 mL 70% cold ethanol, and total DNA was resuspended in 40  $\mu\text{L}$  Tris–HCl buffer, pH 7.2.

### Bacterial community monitoring in the ARID raceway

For bacterial community monitoring in the raceway, the 16SrDNA degenerate primers of (Weisburg et al. 1991) were used to amplify a 1.5 kbp fragment of the 16S rRNA bacterial gene, and incidentally, any 16S rRNA gene fragments of microalgal species that might also be present. The primers were F-5'-AGAGTTTGATCMTGGCTCAG-3' and F-5'-ACGGTTACCTTGTTACGACTT-3'. The amplicons were ligated into the pGEM-Teasy plasmid vector (Promega Inc., USA) and transformed into *Escherichia coli* DH5 $\alpha$  competent cells. Twenty clones selected by colony-PCR that were used to identify clones bearing an insert of the expected size fragment were subjected to bi-directional Sanger DNA sequencing at the University of Arizona Genetics Core (UAGC; <http://uagc.arl.arizona.edu/sanger-sequencing>). The sequences were subjected to BLASTn analysis using the algorithm (Altschul et al. 1990) and sequences available in the NCBI GenBank public database (<https://www.ncbi.nlm.nih.gov>) to identify the closest matches.

## Natural host range study of *V. chlorellavorus* and the algal host in river soil samples

To determine the natural host range of the bacterial predator among the algal species associated with the riverbed, soil samples were collected monthly from February 7, 2014 to January 30, 2015 from the Rillito River bed at a depth of 5 cm and transferred into a sterile 50-mL tube. Soil samples were collected from five sites located an equal distance apart, across a transect spanning the riverbed and its slopes, every third week of each month for 12 consecutive months (2014–2015). Total DNA was isolated by vortex-mixing 3 g soil in 3 mL of Pecos medium followed by a 24-h incubation at room temperature. Particulates were removed by centrifugation at 2000×g. The pellet was resuspended in 3 mL Pecos medium and centrifuged at 8000×g. The final supernatant was used for DNA isolation and purification using the CTAB extraction method described above.

### Primer design for detection and identification of *Vampirovibrio chlorellavorus* and algal community

Based on the 16S rRNA amplicon sequence of *V. chlorellavorus* (KP710184), *V. chlorellavorus*-specific primers, F-5'-GCCAGAGTGGGACTGAGA-3' and R-5'-ACGGCACAGATGGGGTC-3', were designed and used to PCR-amplify a 528-bp fragment of the 16S rRNA gene from total DNA isolated from the riverbed soil samples. The PCR conditions were 95 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s, with final extension at 72 °C for 10 min. To identify the algal community (and possibly other eukaryotes) associated with soil samples collected from the nearby Rillito River, an 18S rRNA degenerate primer was designed based on the consensus sequence of five *Auxenochlorella protothecoides* and 18 *Botryococcus braunii* strains. The primers were F-5'-GGGTTTCGATTCCGGAGAG-3' and R-5'-GTACAAAGGGCAGGGACGTAAT-3', with an expected size of 1.5 kbp in size fragment. The cycling conditions were: 95 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1.5 min, with final extension at 72 °C for 10 min.

The PCR products were resolved by agarose gel (0.8%) electrophoresis in TAE buffer, pH 8.0. The bands were stained with GelGreen Nucleic Acid Gel Stain (Biotium Inc., USA), excised, and gel-purified using QIAquick Gel Extraction Kit (Qiagen Inc.). The amplicons were cloned into the pGEM-Teasy vector (Promega Inc., USA) as described above and transformed into *E. coli* DH5 $\alpha$  competent cells. The plasmid DNA containing inserts of the expected sizes (528 bp and 15 kbp, respectively) was purified from 15 colonies each by colony-PCR (Woodman, 2008). The DNA sequences of the inserts were determined by capillary Sanger sequencing at the UAGC. The provisional identification of community

organisms was assigned out using BLASTn analysis against sequences available in the GenBank database and was based on closest matches, taking into account the similarity score and percent sequence coverage.

### Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out at the Spectroscopy and Imaging Facilities, University of Arizona campus, Tucson. Clean glass slides were prepared by coating with poly-L-lysine to promote cell adherence before applying 200  $\mu$ L of live algal cell culture to the slide. An equal volume of fixative (4% paraformaldehyde and 2% glutaraldehyde in 0.2 M sodium phosphate buffer pH 7.4) was added to each sample, followed by rinsing 3 $\times$  with deionized water and successive dehydration steps with an ethanol series of 15%–30%–50%–70%–90%–100% ethanol. Specimens were mounted and sputter-coated by Hummer 6 Sputtering Device (Anatech Ltd., USA) and imaged using a Hitachi S-4800 Type II/Thermo NORAN NSS EDS: Field-Emission SEM (Hitachi High-Technologies Corp., Japan).

### Decontamination studies

The ARID reactors (946 L) were treated using a 3-day process involving the treatment of all containers, tubing, and water lines with either trichloromelamine (TCM) (Iofina Inc., USA) or Wal-Clean (CH<sub>2</sub>O Inc., Tumwater, WA, USA). The Wal-Clean disinfectant contains a mixture of three surfactants, a sterilizing agent, and a cleanser.

The TCM was evaluated in preliminary tests to treat the ARD reactors and equipment. However, the disinfectant was ineffective for abating *V. chlorellavorus* attack of *C. sorokiniana*, which was evident by the rapid collapse of the outdoor culture, 10 days post-treatment (Fig. 5a). In contrast, treatment with Wal-Clean applied with a foaming machine to produce heavy foam containing 600 ppm chlorine (according to the manufacturer's recommendations) facilitated the growth of algal cultures that were "healthy" and accumulated biomass. On day 1, mechanical cleaning was performed by filling the reactor with water, and increasing the free chlorine level to 10 ppm by the addition of solid sodium dichloro-s-triazinetriene, overnight. On day 2, the reactor water was drained and all surfaces of the reactor were treated with 18.7% trichloromelamine solution using a pressurized spray canister, or by pumping air through a solution of 2.5% Wal-Clean (per manufacturer's suggestion), resulting in a heavy foam, with a minimum contact time of 12 h. On day 3, the disinfectants were rinsed away (2 $\times$ ), and the reactor was filled with water and air pumped through the closed reactor for 12 h to vent volatiles. Finally, Wal-Clean foam was forced through all pipes in the system by connecting to the sealed actively foaming vessel.

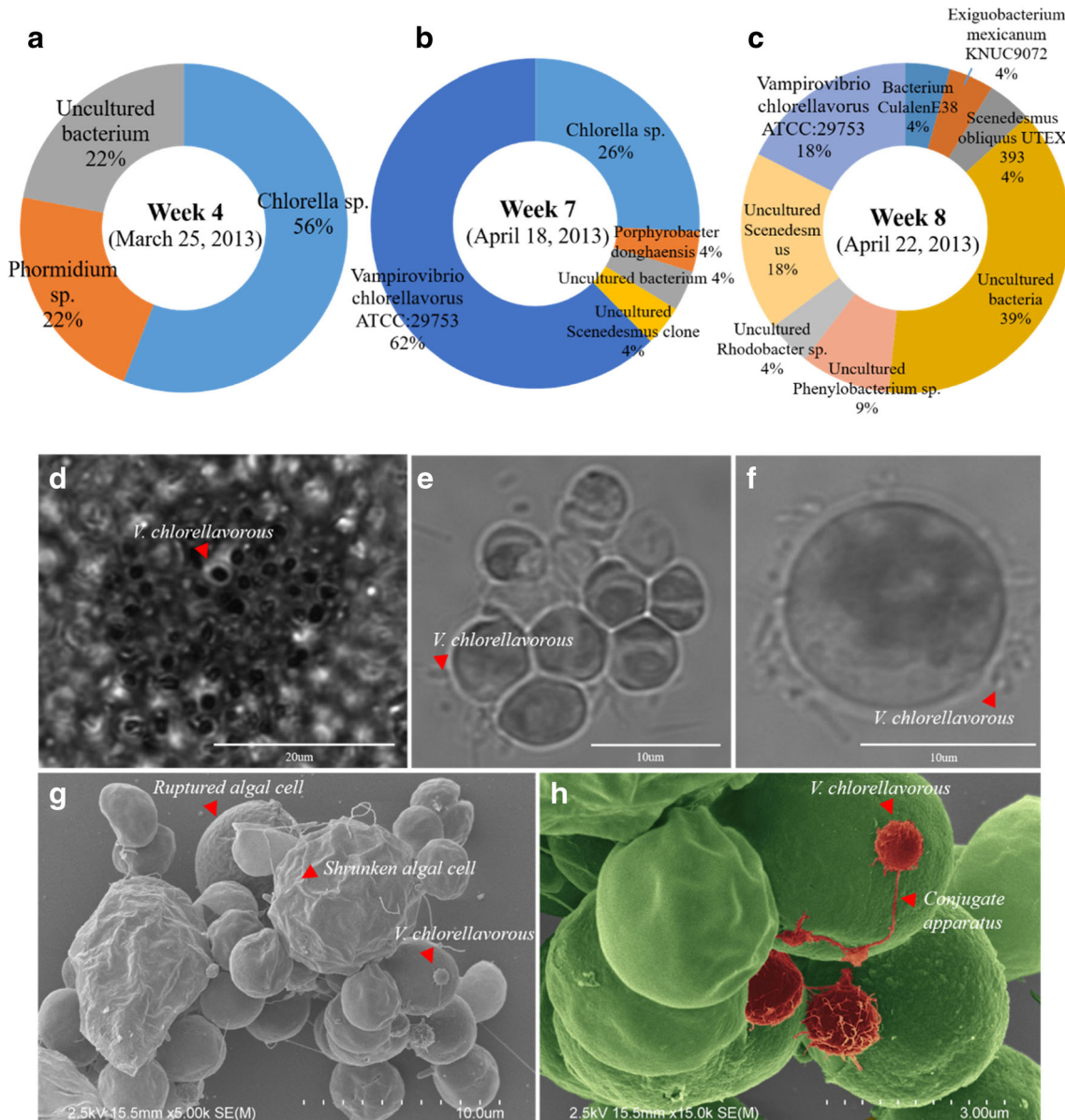


## Results and discussion

### Detection of *V. chlorellavorus* in Arizona ARID raceway in 2013

An analysis of phycosphere samples from the DOE1412 algal cultures under cultivation in the ARID raceway reactor indicated that most of the 16S rRNA gene sequences, e.g., 56% ( $n = 17/32$  of total clones) were algal chloroplast DNA at 4 weeks post-addition of algal inoculum (Fig. 1a and

Supplementary Table S1). By April 18, 2013, the concentration of DOE1412 cells in culture had declined by 24.1% following 23 days of growth to its peak (Supplementary Fig. S1), a trend reflected by the proportion of algal chloroplast 16S rRNA (26%,  $n = 7/27$  of total clones). Accumulation of *V. chlorellavorus* rRNA was first detected along with this decrease in algal culture performance (62%,  $n = 16/27$  of total clones) (Fig. 1b and Supplementary Table S1). The rapid decline of algal cells was also associated with an overall increase in the diversity of the microorganism community (Fig. 1c and



**Fig. 1** Example of an algae raceway integrated design (ARID) reactor phycosphere community analysis, and associated *Vampirovibrio chlorellavorus* cells and its algal host by scanning electron microscopy. **a–c** Circle diagrams showing the fluctuations in the algal associated-bacterial composition based on the abundance of detectable eukaryotic (algal) chloroplast and bacterial 16S rRNA gene sequences. **D–F**,  $\times 40$  (**d**) and  $\times 100$  magnification (**e**, **f**) brightfield micrographs of

*V. chlorellavorus*-infected DOE1412 observed in algal suspension cultures collected from an outdoor reactor (**d**) clumping of cells, characteristic of algal stress (**e**), and a single *Chlorella sorokiniana* DOE 1412 cell (**f**). **e**, **f** Scanning electron micrograph of clumped DOE 1412 with attached *V. chlorellavorus*, at  $\times 5000$  magnification (**g**) and *V. chlorellavorus* (colored purple for contrast)-infected DOE1412, at  $\times 15,000$  magnification (**h**)

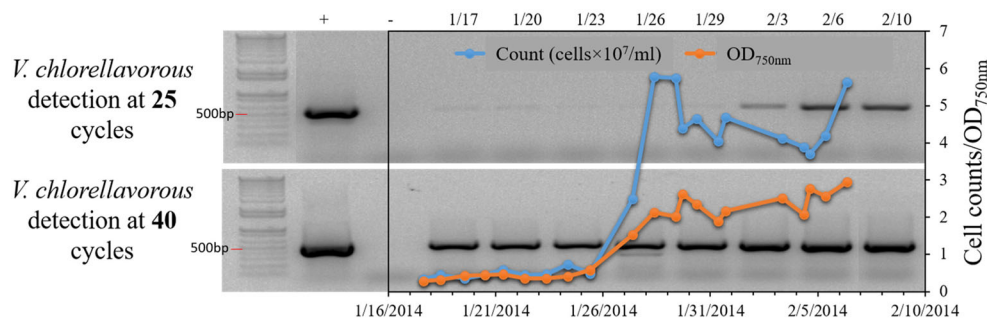
Supplementary Table S1). The shift in prevalence of *V. chlorellavorus* DNA corresponded to a rapid decline in cell count and optical density measurements (Supplementary Fig. S1).

Samples that were found positive by PCR for the presence of *V. chlorellavorus* were observed by light and/or SEM. Algal cells visualized under brightfield light microscopy, with  $\times 40$  and  $\times 100$  magnification (Fig. 1d–f), were light yellow to brown in color, and cells were clumped (Fig. 1d, e). Upon careful inspection, minute particles were observed on the surfaces of the algal cells, an observation that was consistent with previous descriptions of the pathogenic bacteria (Coda and Goff 1986; Gromov and Mamkaeva 1972). For observation at higher resolution, laboratory samples of co-cultured *V. chlorellavorus* and DOE1412 were imaged by SEM. At  $\times 5000$  magnification, the clumped algal cells presented different stages of decline, apparently resulting from bacterial infection, based on the observed cell size and integrity of the cell wall, ranging from cells having an intact cell wall to those showing evidence of having been ruptured and beginning to shrink in size and shrivel, following loss of cytoplasmic and other cellular contents (Fig. 1g). At the highest magnification ( $\times 15,000$ , Fig. 1H), the *V. chlorellavorus* appeared to be attached to its algal host cells by an unknown composition of pad-like structure (highlighted in purple), and in some samples, a fine web-like structure surrounded the bacterial cells and extended to the microalgal host cell wall. The external matrix is involved in the attachment to *Chlorella* cells (Gromov and Mamkaeva 1972).

### Persistence of *V. chlorellavorus* at UAZ ARID site during 2014 cultivation

Monitoring of the DOE1412 suspension cultures in the outdoor ARID raceway for the presence of *V. chlorellavorus* during the 2014 cultivation season using the *V. chlorellavorus*-specific 16S rRNA gene primers indicated nearly year-round presence of the bacterium (Fig. 2). There was one exception during the cultivation period occurring

between May 28 and June 12, 2014 in which an apparently low level of *V. chlorellavorus* was present in the culture but did not compromise *Chlorella* growth, despite being detectable by PCR amplification. An example of a cultivation cycle time-course, demonstrating that the sequential patterns associated with DOE1412 culture establishment, followed by initial growth, and then a rapid decline of the culture, followed by death, was associated with an initial absence of bacterial detection by PCR amplification, followed by a steady increase in detection (upper gel image in Fig. 2). Despite thorough disinfection by manual scrubbing and circulation of 10 ppm sodium dichloro-s-triazinetrione after algal cultivation, the bacterial pathogen was detected by PCR amplification in all of the reactor samples analyzed, when PCR amplification was carried out for 40 cycles, and in only 37% using a 30 PCR cycle protocol. By increasing the PCR cycles from 30 to 40, the limit of detection of *V. chlorellavorus* was increased substantially. This permitted detection of the bacterium during the cooler time of the year, from January 29 to February 3, previously a timeframe during which the bacterium is not undetected in the reactors. Following this early-season detection of *V. chlorellavorus*, it became possible to attribute the culture crash to bacterial infection, when the algal cell density measurements declined substantially on February 7 and signaled the impending decline (Fig. 2). The rapid decline in the algal cell count numbers and OD<sub>750</sub> values were closely associated with the PCR detection profile of *V. chlorellavorus*. Furthermore, *V. chlorellavorus* was found to have been present almost immediately after the raceway was inoculated with the algal culture, based on molecular analysis of water samples collected daily from the raceway. Although apparently the bacterium was present at undetectable levels during the first few days of sampling, the population size increased rapidly to detectable levels within several days, resulting in rapid decline and death of the algal culture, and leading to a total loss of the run. Similar trends were observed in DOE1412 cultivation runs throughout the winter and summer seasons during 2014 (Table 1), indicating the bacterium has become a chronic problem and impediment to cultivation of



**Fig. 2** Detection of the *Vampirovibrio chlorellavorus* 16Sr RNA gene, in relation to *Chlorella sorokiniana* DOE1412 growth in the ARID raceway from January 17 to February 10, 2014. Growth of DOE1412 was quantified based on cell counts and optical density at 750 nm. Note:

“+” depicts PCR positive control (10 ng cloned *V. chlorellavorus* 16sRNA), and “–” indicates the negative PCR control consisting of double-distilled dH<sub>2</sub>O instead of DNA template

**Table 1** Detection of *Vampirovibrio chlorellavorus* in algae raceway integrated design reactors during 2014. Total genomic DNA was isolated from raceway algal suspension culture and used to PCR-amplify a

fragment of the *V. chlorellavorus* 16S rRNA gene, whose identity was verified by cloning and DNA sequencing. The abbreviation “PW” indicates paddle wheel raceway

Start date	End date	<i>V. chlorellavorus</i> positive/detection attempts			Detection rate (%)
		ARID	PW1	PW2	
Jan. 17	Feb. 10	8/8	N/A	N/A	100
May 28	Jun 12	0/3	0/3	0/3	0
Jun 13	Jun25	2/3	2/2	2/2	86
Jun 30	July 23	3/3	2/2	2/2	100
Aug. 5	Aug. 20	3/3	1/3	1/3	56
Oct. 15	Nov. 20	4/5	4/5	5/5	87

DOE1412 at the ARID reactor test site. The rapid shift of *V. chlorellavorus* to a predatory lifestyle is closely related to the water temperature (Li 2015). The study showed that the bacterial population dramatically increased at the water temperature ranging from 28 to 34 °C, heavily infesting *Chlorella* algal host. Under extreme temperatures at 4 and 41 °C, *V. chlorellavorus* remained avirulent.

**Source of *V. chlorellavorus***

Soil samples were collected from the Rillito River between February 2014 and January 2015 to examine the proximal origin of *V. chlorellavorus*. The sampling site was chosen as the habitat nearest the ARID raceway (130 m north) likely to harbor the pathogen, as the normally dry river basin experiences heavy flooding in the summer along with more gentle intermittent flows during winter months (Tadayon et al. 1995).

Sampling sites form a cross section of the riverbed range from dry sandy soil to moist clay containing visible green deposits. Despite dramatic shifts in temperature and soil moisture throughout the year, *V. chlorellavorus* detection by specific 16S rRNA PCR was ubiquitous, 100% detection in all sampling sites in April with only November resulting in a rate under 85% (Table 2). This was surprising as the pathogen’s host range has been reported to be restricted to the genus *Chlorella* (Coder and Goff 1986), which would be severely inhibited by the prolonged periods of desiccation (Holzinger and Karsten 2013). The findings open the possibility of the presence of an alternative natural host for *V. chlorellavorus*. A previous study showed that a wide range of soil algae has occurred in the Sonoran desert in Arizona that includes *C. vulgaris* which is an algal host for the *V. chlorellavorus* (Cameron 1960), and many of them can be existed during long periods of desiccation (Trainor 1985). Another

**Table 2** Detection of *Vampirovibrio chlorellavorus* from soil samples collected in the Rillito River, Tucson AZ. Soil samples were collected from five sites located an equal distance apart and across a designated transect, respectively, spanning the riverbed and its slopes, every third

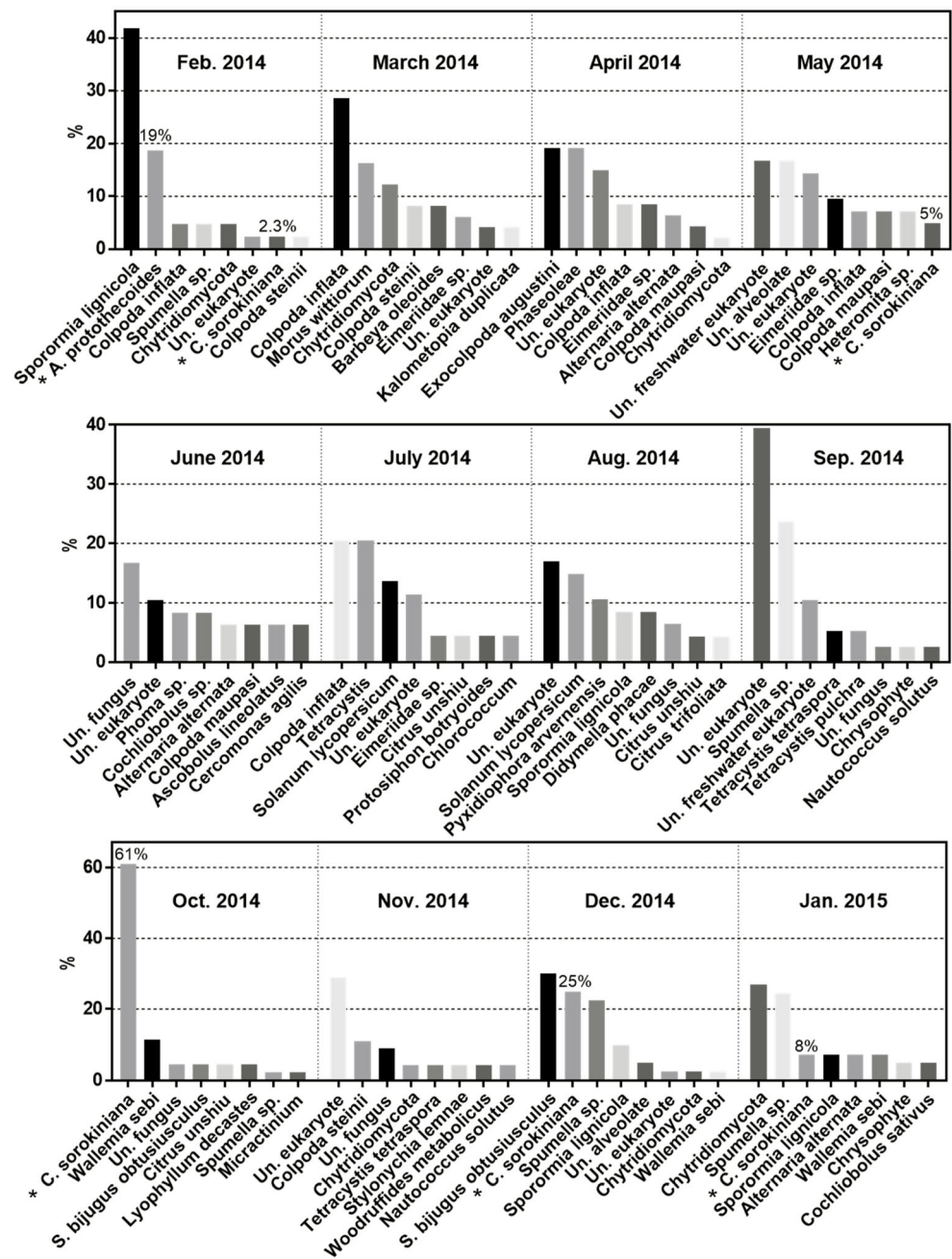
week of the month for 12 consecutive months (2014–2015). Total DNA isolated from soil samples was used for PCR detection of *V. chlorellavorus* using species-specific primers designed to amplify the 16S rRNA gene



Month	<i>V. chlorellavorus</i> positive / Detection attempts					Detection Rate (%)
	Site 1	Site 2	Site 3	Site 4	Site 5	
2014 Feb.	4/4	3/4	3/4	4/4	2/4	80
Mar.	4/4	2/4	4/4	3/4	4/4	85
Apr.	4/4	4/4	4/4	4/4	4/4	100
May	3/4	3/4	3/4	4/4	4/4	85
Jun.	4/4	3/4	3/4	4/4	4/4	90
Jul.	3/4	4/4	4/4	4/4	4/4	95
Aug.	4/4	4/4	3/4	4/4	4/4	95
Sep.	4/4	4/4	4/4	3/4	4/4	95
Oct.	4/4	4/4	4/4	2/4	3/4	85
Nov.	4/4	2/4	2/4	2/4	3/4	65
Dec.	4/4	4/4	3/4	4/4	4/4	95
2015 Jan.	4/4	4/4	4/4	4/4	4/4	100



**Fig. 3** Natural algal host range study based on PCR amplification and DNA sequencing of the cloned 18S rRNA sequence for eukaryotic organisms detectable in soil samples collected from the Rillito River, immediately adjacent to the cultivation facility, shown as percentage of the total sequences determined, for each sampling month. The previously documented algal species known to serve as a host of *V. chlorellavorus* are marked with asterisk. Note: the abbreviation for “uncultured” is indicated as “un”



possibility would be the presence of a resting form of *V. chlorellavorus*, but this has not been reported yet.

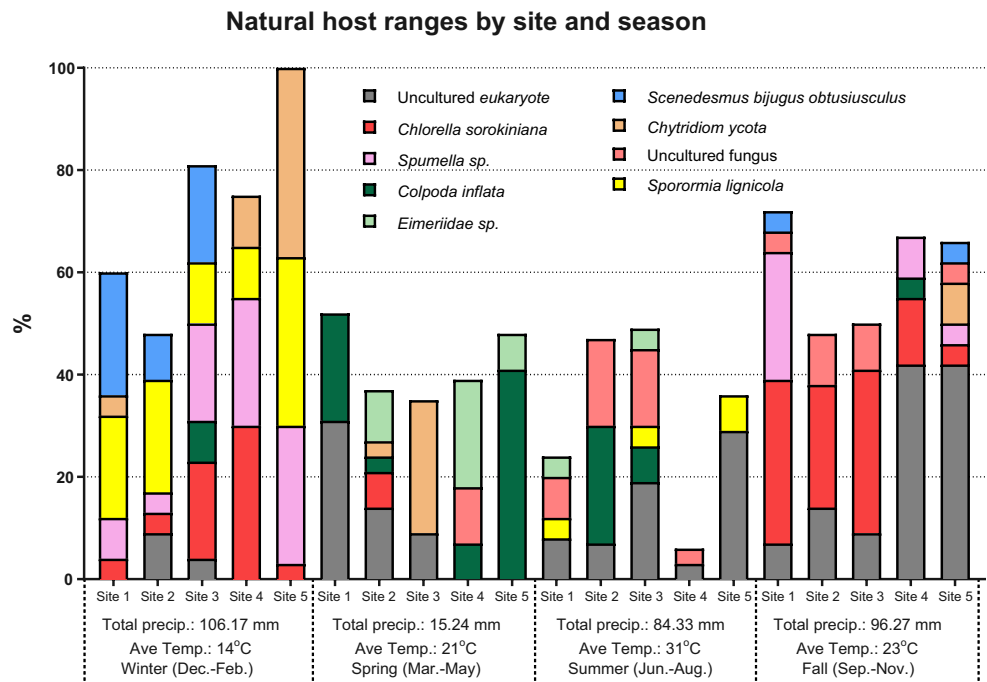
A variety of eukaryotic microbes were detected using 18S rRNA degenerate primer from the five sites sampled from the Rillito River between February 2014 and January 2015 (Fig. 3). Notably, the desiccated river soil contains DNA of several algal species, *C. sorokiniana*, *Auxenochlorella protothecoides*, *Tetracystis*, and *Spumella*, which are well-known regular members of soil flora (Metting 1981). Among the sequence descriptions, *C. sorokiniana* is the only known host of *V. chlorellavorus* (Coder and Goff 1986) and is conspicuously missing from seven of the 12 months sampled.

During February and May, *C. sorokiniana* represented 2.3 and 5% of detected sequences respectively. Following the July–August monsoon season, the algal host was enriched during the fall season in the October, December, and January samples at 61, 25, and 8%, respectively (Fig. 3).

In addition to the *Chlorella* species, a number of eukaryotes were also identified in the samples collected in the Rillito River soil including *Spumella* sp., an uncultured fungus, and *Chytridiomycota* (Fig. 4). The identification was achieved based on the sequence similarity of the PCR-amplified 18SrRNA gene collected in the soil sampling sites by comparing to NCBI GenBank. The phototrophic flagellate,

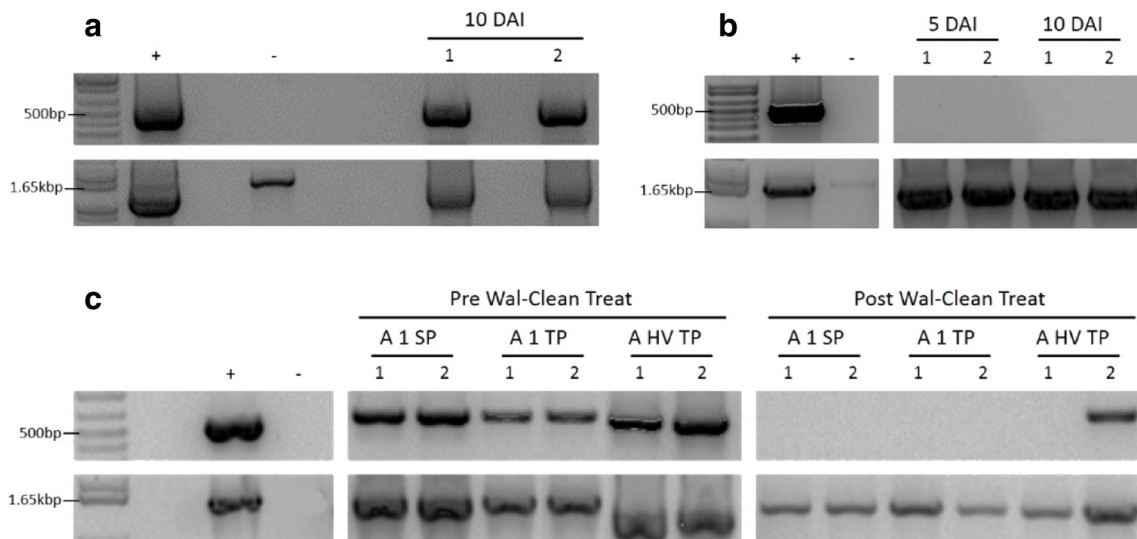


**Fig. 4** Natural algal host range for *Vampirovibrio chlorellavorus*. The study was based on PCR amplification and DNA sequencing of the 18S rRNA sequence for all detectable eukaryotic organisms in soil samples collected from the Rillito River shown as percentage of total sequences obtained for each sampling date



*Spumella* sp., was detected throughout the sampling period, a species implicated as a primary bacteria grazer in pelagic systems with a role in transferring carbon to higher-level organisms (Holen and Boraas 1991). The presence of the *Spumella* sp. in the soil samples suggests that such genera might regulate the *V. chlorellavorus* population. In addition, the aquatic fungi, *Chytridiomycota*, known to be an algal parasite (Ibelings et al. 2004), were associated with the river soil

samples. A certain chytrid, *Rhizophyidium scenedesmi*, is an epibiotic parasite that feeds on *Chlorella* (Gromov et al. 1999) and *Graesiella* sp. (previously known as *C. emmerstonii*) (Ding et al. 2018). The devastating effect of a chytrid in the commercially valuable green microalga *Haematococcus pluvialis*, a natural source of the carotenoid pigment astaxanthin, was reported (Strittmatter et al. 2016). Other common soil ciliates such as *Colpoda* sp., frequently detected



**Fig. 5** Efficacy of disinfectant treatments based on PCR detection of *Vampirovibrio chlorellavorus* DOE1412 16S rRNA gene (label shows location of 500 bp expected size band) and bacterial 16S rRNA gene (label shows location of 1650 bp expected size band). **a** Efficacy of trichloromelamine (TCM)-treated water samples collected from the 946-L closed reactor, 10 days post-inoculation (DAI) with DOE1412, based on PCR results. **b** Effect of Wal-Clean treatment assayed by PCR

of samples taken from the 946-L closed reactor 5 and 10 DAI. **c** Effect of Wal-Clean treatment determined by PCR detection of *V. chlorellavorus* in samples collected from the reactors, the 1st stand pipe (A1SP), the terminal pipe (A1TP), and the HV terminal pipe (AHVTP). Note: “+” depicts PCR positive control (10 ng of cloned *V. chlorellavorus* 16sRNA), and “-” depicts PCR negative control (ddH<sub>2</sub>O). Numerals 1 and 2 indicate replicated samples

in the riverbed soil, are known to form resting cysts that confer tolerance against freezing and desiccation, and to feed on a wide range of bacteria (Ruthmann and Kuck 1985; Muller et al. 2010). The taxonomic profiles of eukaryotic 18S rRNA gene in the five riverbed sites sampled were indicative of dynamic shifts occurring in response to the season and particular sampling site (Fig. 4). Drastic changes in taxonomic profiles is known to be caused by large influxes of soil algae and microbes that are dispersed by the air, water, and even by different kinds of organisms (Kristiansen 1996). The sites were characterized as having different vegetation profiles across the riverbed, with the slopes and edges of the riverbed (site 1, 2, 4, and 5) supporting shrubs that provide shade and soil stability, resulting in a moist environment that is favorable to algal growth. In contrast, site 3 was quite dry owing to the presence of primarily gravel and sand, and the absence of vegetation. The PCR results indicated that *C. sorokiniana* was only detectable during the rainy and wet times of the year, typically experienced in this location during the fall and winter seasons. In particular, it was detected primarily in the moist, least disturbed locations at sites 1 and 4. Collectively, these results indicate that the riverbed soil supports a myriad of prokaryotic- and eukaryotic organisms, as expected for a healthy open ecosystem continuously receiving an influx of organic matter and large microbial load from environment.

### Disinfectant treatment

The algal culture inoculum produced for the ARID raceway was routinely scaled-up in 946-L closed reactors and pumped through transfer pipes into the reactor. When tested by PCR, *V. chlorellavorus* was present in all of the containers, pipes, and tubing used in the post-inoculation cultivation steps (Fig. 5a, c). Laboratory, scale-up, and field procedures to increase sanitary practices and treatment with selected chemical disinfectants were investigated to determine the efficacy to reduce or eliminate *V. chlorellavorus* from all components of the system. Together with the routine use of swimming pool grade chlorine (sodium dichloro-s-triazinetriene), TCM was evaluated as a potential disinfectant that was applied to the reactors and transfer pipe lines. This compound is a bactericidal halamine previously used for emergency sterilization (Worley and Williams 1988). When it was used to coat the interior surfaces of the ARID large and paddlewheel reactors, the *V. chlorellavorus* 16S rRNA gene persisted, based on detection by PCR amplification in the DOE1412 cultures, post-inoculation of the reactors (Fig. 5a). Consequently, Wal-Clean, another commercial disinfectant, was tested as a TCM replacement. The active sterilizing agent in Wal-Clean is sodium hypochlorite, which is a strong oxidizing agent delivered in the form of hypochlorous acid (HOCl) and hypochlorite ion ( $\text{OCl}^-$ ), in equilibrium, depending on pH. Hypochlorous acid has been shown to be an effective

bactericide because it diffuses through the bacterial cell membrane where it is toxic to the cell (Fukuzaki 2006). Of the two compounds tested, treatment with Wal-Clean proved to be the superior disinfectant, based on PCR detection in samples collected from the reactor and the associated components of the cultivation system, 5 and 10 days post-inoculation of the reactor with *V. chlorellavorus*-free DOE1412 lab cultures (Fig. 5b). In addition to cleaning surfaces, the foam was highly effective at scouring the crevices, curves, and other recalcitrant locations within the transfer pipe lines where the bacterium was shown to be capable of residing (Fig. 5c). The results reported herein for Wal-Clean disinfection efficiency of the reactors and transfer lines are consistent with those reported in a previous study, in which bacterial counts were reduced by several orders of magnitude compared to treatment with sodium hypochlorite (NaOCl) alone (Chaidez et al. 2003).

### Conclusions

In this study, the presence of year-round, naturally occurring, *V. chlorellavorus* was monitored during 2014–2015 to better understand the dynamics of the predatory bacterium in an arid-climate outdoor reactor and the extent of the economic losses to algal production. Here, the monitoring results showed a tight association between *V. chlorellavorus* and one of its several known microalgal hosts, *C. sorokiniana*, resulting in rapid decline and death following attack, which was consistent with the results of the molecular technique that was developed and applied for monitoring during this study. Within the ARID cultivation system, the foaming chlorine treatment facilitated the most effective disinfection of the system and its components, which eliminated or greatly reduced detectable bacterial levels when used to clean the system regularly between cultivation runs. The prevalence of *V. chlorellavorus* predation on the microalgal host, *C. sorokiniana*, at the ARID reactor site (Tucson AZ USA) was greatest during the fall and winter seasons, and in particular, when precipitation was recorded. The onset of disease requires compatible interactions between the host, pathogen, and environment. The results of this study demonstrate that at least two of the three factors essential for a disease outbreak to occur were consistently met in the ARID reactor; however, the environmental factors that triggered the attack and demise of the algal host(s) are largely unknown, except for the observed correlation with rainfall and gusty conditions, suggesting they are likely modes of inoculation of the reactors by naturally occurring *V. chlorellavorus*. In addition, routine analysis of environmental samples collected at and near the ARID reactor site for *V. chlorellavorus* presence by PCR amplification further showed that the DOE1412 cell death rate was highest when daytime water temperatures reached 32–35 °C, strongly implicating temperature as a trigger of successful attack and

therefore of susceptibility of the algal host to the pathogen (Li 2015). Consistent with these combined factors, outbreaks of *V. chlorellavorus* did not occur when the reactor water temperature remained below 32–35 °C and in the absence of rainfall. However, temperatures in this same range are known to favor the growth of *C. sorokiniana*, and therefore, it seems likely that rapid algal growth may be an essential requirement for successful predation by *V. chlorellavorus*. Whether this requirement is the result of direct or indirect effects is not yet known. The year-round detection of *V. chlorellavorus* from the nearby Rillito River soil samples suggested that the source of predatory bacteria is from the riverbed soil. The survival was possibly enabled by the availability of one or more algal hosts, including *C. sorokiniana* which was detected in river bed soil samples where *V. chlorellavorus* was also detected. Among sampling times, the algal host was found to be highly enriched following the seasonal monsoon rains, in samples collected during October, in which 61% of the samples were positive for the algal host.

Several parallel strategies to protect the outdoor ARID cultivation system from microbial invaders can be considered. First, establishing growth conditions to enable the maintenance of the cultivation system at temperatures known to be most unfavorable for growth of the bacterial pathogen is an important proactive strategy to achieve optimal biomass production. Second, it is essential to disinfect the cultivation system and its components on a regular basis, after each cultivation run, with effective compounds such as a chlorinating agent together with the commercially available disinfectant, Wal-Clean. And third, the routine uses of a molecular detection tool to provide surveillance of the cultivation system, from the laboratory cultures, during scale-up, and post-inoculation of the reactors, together with close monitoring of environmental conditions, can minimize risk of *V. chlorellavorus* attack. In the ARID system, deployment of an integrated approach was shown to facilitate production of an otherwise *V. chlorellavorus*-susceptible microalgal species during the spring, summer, and fall seasons at the Tucson, AZ reactor site. Although the eradication of this apparently naturally occurring *V. chlorellavorus* is not likely, by combining precautionary efforts including real-time monitoring of phycosphere community composition using molecular tools, which are more sensitive than was detectable using the light microscope for monitoring, it was possible to capitalize on the ability to detect this damaging pathogenic bacterium early, prior to the onset of symptoms (cell death and culture decline). Thus, protective measures can be taken to ensure that *C. sorokiniana* biomass could be produced in harvestable quantities, even though this commercially favorable microalga is among the most preferred hosts of *V. chlorellavorus*.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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