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ABSTRACT

The environmental conditions created in the Southwestern deserts of the United States are conducive to the production of green microalgae biomass, for use as a feedstock intended for conversion to carbon neutral liquid biodiesel. One promising heat-tolerant, rapidly-growing, high lipid content species is the chlorophyte, *Chlorella sorokiniana* (Shihira and Krauss, 1965) (isolate DOE 1412), which has been selected for pilot-scale production as part of a larger algal biofuels project to assess its potential for long-term productivity in open, outdoor monoculture production systems. Molecular analysis exposed the presence of the pathogenic bacterium, *Vampirovibrio chlorellavorus* (Gromov & Mamkaeva, 1972) causing infection and death of DOE 1412, which occurred most rapidly at air temperatures exceeding 34 °C. This Gram-negative bacterium has been reported to attach to and utilize the cellular contents of several *Chlorella* species, leading to yellowing and flocculation of algal cells, and death of the host. A quantitative PCR assay was developed to monitor pathogen accumulation using the 16S ribosomal RNA gene, in addition to the algal 18S ribosomal RNA gene for normalization. The assay is highly sensitive, with limits of quantification for the 16S and 18S gene targets calculated to be 19 and 131 copies, respectively. The qPCR assay was used to monitor several outdoor reactors inoculated with the DOE1412, throughout the warm season growth-to-harvest cycle to understand the disease cycle and inform disease management decisions. Further, the bacterium was monitored in paddlewheel DOE 1412 cultures treated with benzalkonium chloride (BAC), a biocide tested for the ability *V. chlorellavorus* attack of DOE 1412. The treatment resulted in a reduced growth rate for DOE 1412, but prolonged the duration of the production cycle resulting in increased total harvestable yield, compared to untreated control cultures.
INTRODUCTION

1.1 Literature review

1.1.1 Microalgae mass cultivation for biofuel production

Single celled green microalgae have been the subject of investigation and culturing at various scales since as early as 1850, when reports were published on the growth of *Haematococcus sp.* in laboratory cultures for an extended period. Since that time, the characterization and cultivation of algal species has proven fruitful for research and industrial applications (Andersen, 2005). Mass culture of microalgae has been established as a commercially viable endeavor for
the production of high-value products such as carotenoids (Del Campo et al., 2007), long-chain polyunsaturated fatty acids (Mendes et al., 2008), and phycobilins (Singh et al., 2005). The earliest reports on the concept of utilizing microalgae for the large scale production of fats, in the form of triacylglycerols (TAGs), date back to mid-twentieth century Germany (Harder and von Witsch, 1942). Since that time concerted efforts to study algal biofuels have largely followed political and economic trends in energy policy and production (Borowitzka and Moheimani, 2013), which prompted the initiation of the United States Department of Energy’s Aquatic Species Program (ASP). During the ASP, over 3000 microalgae strains were screened for various relevant characteristics (i.e. pH, salinity, and temperature tolerances), lipid biosynthesis physiology was investigated, genetic engineering studies were completed, and outdoor production was demonstrated (Sheehan et al., 1998).

Continued interest in microalgae as a biofuels feedstock has yielded a substantial body of evidence in support of the process. A survey of over 100 green microalgae studies found strains to have an average of 25.5% lipid content of dry cell weight (DCW) under optimal growth conditions, and an average of 45.7% DCW lipid content under nutrient-depleted conditions (Hu et al., 2008). Several reports indicate that *Chlorella* species grown under nitrogen deplete conditions accumulated as much as 85% of their DCW as lipid molecules (Iwamoto et al., 1955; Spoehr and Milner, 1949). Photoautotrophic microalgae grow quickly, with some doubling as much as five times per day (Goldman and Carpenter, 1974). Unlike biofuels derived from cellulosic plant biomass, microalgae cultivation can be successfully performed on non-arable and marginal lands, reducing competition for precious crop production land resources (Singh et al., 2011). In addition to light, requirements for algal growth can be met in sustainable ways. The water required for cultivation can be recycled upon biomass harvest, and many species have been
shown to thrive in wastewater, utilizing excess nutrients while sequestering contaminants (Pittman et al., 2011). Importantly, microalgae cells fix atmospheric CO$_2$, which can also be obtained from co-located sources such as traditional power plants (Maeda et al., 1995). Despite positive aspects of algae biofuels, financial models that are based on currently reported data indicate that production costs are much higher than those of traditional petroleum liquid fuel, and that increased efficiency will be required at multiple stages, with respect to strain selection, nutrient/water inputs during production, harvesting, and predators and pathogen management (Slade and Bauen, 2013).

1.1.2 *Chlorella sorokiniana*: selecting an ideal desert dweller

The body of literature on microalgae growth parameters, as well as environmental and economic factors indicates that improvements are required in current practices, to reach a sustainable biomass productivity goal of 30 g m$^{-2}$day$^{-1}$ (U.S. Department of Energy, Multi-year Program Plan, 2014). A core requirement in algal biofuel production is maximized lipid productivity, or combined biomass productivity, expressed as dry algal mass/area/time, and lipid content measured as percent lipid/dry algal mass. In addition to operational strategies, the ASP report indicated that the strategic choice of microalgae strains best-suited for selected environments at each algal farming site is critical to meeting production goals (Sheehan et al., 1998). This has led to the study and selection of algal species that exhibit optimal biomass yield and lipid content when cultivated under variously specified conditions, including high carbon dioxide concentrations for improved carbon sequestration (Yoo et al., 2010), and high saline conditions for exclusion of competitors (Montero et al., 2010).

After outlining beneficial factors for algal growth in the Southwest region of the United States (McIntosh, 1985), large field collection and screening programs were conducted to isolate
suitable strains (Sommerfeld, 1986). Modeling using a biomass assessment tool based on national datasets of environmental parameters found that a field isolate of *Chlorella sorokiniana* (designated DOE1412, isolated from surface water in Texas by Dr. Juergen Polle of Brooklyn College) thrives in high temperature and light intensity conditions. The maximum growth rate of DOE1412 was measured at 36°C before dropping at 41°C, making the strain an ideal performer in the targeted locations where culture temperatures routinely surpass 33°C during the summer months (Huesemann et al., 2013, 2016).

1.1.3 Ecology and physiology of microalgae culture invaders

The term phycosphere, analogous to the rhizosphere concept, indicates a physical zone of varying dimensions wherein extracellular products of algal cells stimulate the growth of associated bacterial species (Bell and Mitchell, 1972). It has since been expanded to encompass all algal-microbial biotic interactions (Cole, 1982). Positive, and indeed, obligatory interactions within the phycosphere are well documented, such as the dependency of many microalgae species on bacteria for the production of the essential vitamin B12 (Croft et al., 2005). Antagonistic relationships within the phycosphere have also been documented to include fungi, amoeba, bacteria, viruses, as well as a wider diversity of microbes acting by various mechanisms as parasites of algae species grown in mass culture (Carney and Lane, 2014). The most commonly reported fungal parasites of microalgae come from the Chytridmycota phylum, and have been observed as both saprotrophs and parasites in natural fresh waters (Kagami et al., 2011). The chytrid fungus, *Phlyctidium scenedesmi*, was identified in a mass culture system growing *Scenedesmus sp.* green microalgae and caused growth operations to be halted due to its destructive impact (Fott, 1967).
While the importance of the above organisms should not be discounted, the most abundant non-algal members of both natural and artificial phycospheres belong to the bacterial kingdom. Thus, the interactions between bacteria and algae species have been studied extensively and found to interact in beneficial, antagonistic, and neutral fashion (Cole, 1982; Ramanan et al., 2016). A study on seasonal algal blooms (analogous to mass culturing in open reactors) found that peaks in phytoplankton abundance were immediately followed by accumulation of bacterial cells to densities as high as $13\times10^6$ cells ml$^{-1}$ by direct microscopic counts of a freshwater lake (Coveney et al., 1977). As the primary producers in most of the ecosystems in which they reside, microalgae growth and senescence attracts many bacterial species to metabolize the resulting detritus as well as those specialized for parasitism on the living algal cells directly. The most commonly reported mechanism of microbial parasitism on microalgae entails bacterial cells in close proximity to their algal targets excreting cell wall degrading enzymes including glucosidases, chitinases, and cellulases. Bacteria belonging to the genera *Acinetobacter*, *Ruegeria*, and *Pseudomonas* were each isolated from cultures of the marine microalgae *Tetraselmas indica*, and were observed by scanning electron and epifluorescent microscopy in close association with cell walls, while also exhibiting degradation of pertinent polysaccharide compounds (Arora et al., 2012). Similarly, study of *Alexandrium tamarense* microalgae found that *Alteromonas sp.* and *Thalassobius aestuarii sp.* bacteria accumulated on algal cell walls, leading to lysis and more than 50% decrease in chlorophyll $a$ content compared to un-inoculated controls. These bacteria also caused a significant increase in the activity of both $\beta$-glucosidase and chitinase enzymes when added to *A. tamarense* cultures (Wang et al., 2010). Algicidal bacteria exhibit a variety of known mechanisms for initiation of their various parasitic life cycles. Motility is a common phenotype among aquatic bacteria adapted to symbiosis with
microalgae and can be accomplished by flagella, but most studies on specifically parasitic
bacteria have focused on a ‘gliding’ mechanism whereby cells move slowly along a mucilage
deposit on solid surfaces (Daft et al., 1975). Such movement has been observed in Cytophaga
sp. parasitizing marine phytoplankton (Imai et al., 1993), and excellently described by study of
Saprospira sp. bacterium invading the cytoplasm of its diatom host. The gliding motility was
found to be dependent on the formation of microtubule structures within the bacterial cells
(Furusawa et al., 2003). Another critical physiological aspect of bacteria seeking to consume
algal products is the sensing of chemical signals to illicit chemotactic response. Chemotactic
response correlated positively with algae culture age, as described by measuring the movement
of bacteria towards total microalgae filtrate (Bell and Mitchell, 1972). Investigation of the
phytoplankton-produced compound, dimethylsulfoniopropionate (DMSP), found that strong
chemotactic response was induced in heterotrophic bacteria at concentrations well below
amounts produced in natural algal ecosystems (Seymour et al., 2010). Additionally, there is
convincing evidence that algicidal bacteria can switch to parasitic activities by quorum sensing
activation of the alternative induction pathway (AI-2) in response to microalgae growth (Skerratt
et al., 2002). Despite all of this research on the subject, there remains a great deal of mystery to
parasitism of bacteria on microalgae and the specifics of V. chlorellavorus activity (described
below) will demonstrate a number of mechanisms novel to this particular field of study.

1.1.4 Methods for detection and quantification of microorganisms

Given the preponderance of invasive species in a variety of open culture systems (section 1.1.3),
special attention is now being given to methods for the identification and characterization of bio-
contaminants (Carney and Lane, 2014; Slocombe et al., 2016). Microscopy has long served as
the standard for both identification and quantification of microorganisms in mixed samples
(Rasconi et al., 2009). While this approach still offers benefits in the speed of analysis (can be performed within minutes or hours of sampling) and in the morphological identification of novel invasive species, it has shortcomings for routine monitoring. Microscopy can become labor intensive with increasing numbers of samples to be analyzed due to low throughput and the requirement of expert knowledge for effective identification of contaminants (Slocombe et al., 2016). The assessment of bio-contaminants for industrial scale production demands automated techniques that can process samples at a greater rate, while decreasing the ambiguities of visual assessment. The latest methods employed for this task have included the use of digital image analysis of flow cytometry (Álvarez et al., 2011) as well as confocal fluorescence microscopy to detect minute fluctuations in signature algal auto fluorescence caused by biological contaminants (Collins et al., 2014). These advanced optical techniques improve upon the classic cell counting methods, but still rely on previous knowledge of harmful pests.

Molecular detection methods include a variety of techniques that may be more or less effective given the particular system. The most effective molecular monitoring schemes consist of a two phase approach wherein sequencing of conserved genetic ‘barcoding’ regions is used as a screen for potential problem contaminants, followed by the development of more rapid assays to target the putative etiological agent(s). The first phase can be addressed by a non-targeted method of culture free amplification of barcode regions such as the large and small subunits of the ribosomal RNA gene (LSU and SSU rRNA) or the internal transcribed spacer (ITS) region of the same gene (Boenigk et al., 2012). Total genomic DNA representing the whole phycosphere community of a diseased algal sample can be used as a template for degenerate PCR primers targeting one or more fragments of these regions, which can then be sequenced by high throughput next generation instruments. For instance, the sequencing of a 250 bp fragment of
informative SSU rRNA can be performed on over 30,000 unique samples at a depth of 10,000 reads using the Illumina HiSeq2000 machine, allowing for the quantitative assessment of bacterial DNA and genus level taxonomic estimation (Caporaso et al., 2012). Taxa, which are enriched in diseased microalgae cultures can then become candidates for the second phase of detection. Alternatively, cultures displaying disease symptoms can be collected and pest organisms can be isolated for more specific identification by standard PCR amplification and Sanger sequencing of barcode DNA. McBride and colleagues employed this strategy to identify putative Cryptomycota which were forming plaques on agar plates coated with the chlorophytic algae, Scenedesmus dimorphus. The same study carried the molecular monitoring scheme to the second phase by designing a specific assay, based on quantitative PCR (qPCR), for the detection of the invasive species (McBride et al., 2014). Molecular diagnostics for specific pests can be accomplished by a few general methods. Microarrays can be very useful for the simultaneous detection of many pre-determined nucleic acid targets in a short time frame, but suffer from laborious probe design and relatively low sensitivity, often demanding additional enrichment steps (Binga et al., 2008). Fluorescent in situ hybridization is another possible method for detection and accurate quantification of many microorganisms, and has been used to effectively detect epiphytic bacteria associated with marine microalgae (Tujula et al., 2006). The method can be extremely sensitive and accurate, but suffers from the same issues of light microscopy in terms of throughput.

The favored methodology for detection and enumeration of microorganisms is qPCR, which can be performed by two general approaches. The first relies on DNA intercalating dye, often SYBR green, to report the amplification of transcripts during PCR by emitting fluorescence upon binding to nascent double stranded DNA (Ponchel et al., 2003). This type of assay can be very
useful for the quantification of DNA under the proper conditions and is the least costly of the options, but it does rely heavily on the specificity of the design of standard PCR primer sets. The second option is the utilization of hydrolysis probe based systems, whereby a probe sequence is designed to complement a target sequence nested within forward and reverse primer sites, and is bonded to a fluorescent molecule that is quenched by and adjacent bound chemical group with compatible light absorption properties. These two molecules are separated during each round of PCR amplification by the hydrolytic activity of DNA polymerase, releasing the indicative and quantitative signal of light (Holland et al., 1991). In practice, this method has yielded positive detection of target DNA sequences diluted down to fewer than 10 copies (Boyle et al., 2012). The requirement for the probe annealing site in addition to the standard PCR primers lends an extremely high degree of specificity, with researchers readily able to differentiate 6 groups amongst a suite of very closely related archaeal strains even within the highly conserved 16S rDNA sequence region (Yu et al., 2005). The technique has also proven effective in the enumeration of marine microorganisms in aquatic environments as demonstrated by studies on harmful algal blooms (Yuan et al., 2012; Zhang et al., 2015). One objection to the use of qPCR, and other culture free detection methods, is the inability to meet Koch’s postulates for identifying an etiological agent of disease. Attempts to revise these widely accepted stipulations with the realization that the majority of microbes are currently ‘un-culturable’ may preclude the possibility of meeting the requirement for independent cultivation of a putative causative microorganism. Acceptance of modified criteria are becoming more commonplace as the use of culture independent methods of identification and quantification continues to grow (Fredericks and Relman, 1996).

1.1.5 *Bdellovibrio* and Like Organisms (BALO)
The dynamics of *Vampirovibrio chlorellavorus* and its interaction with algal host cells are most similar to a better studies group of bacteria known as *Bdellovibrio* and like organisms (BALOs). The first description of this group was published in the 1960’s and established this unusual set of predatory, inter-periplasmic bacteria (Stolp and Petzold, 1962). Since that time the BALOs have been expanded to include several distinct genera of bacteria that all prey upon other Gram-negative bacterial cells. It is no coincidence that early publications on *V. chlorellavorus* were made by leading BALO researchers, as initial descriptions lead to misidentification as a member of the group (Jurkevitch and Davidov, 2006). While it was subsequently reclassified, the relative paucity of publications on *V. chlorellavorus* specifically leaves the BALOs as an excellent source of information of behaviorally similar organisms (their periplasmic invasion strategy is the primary factor setting them apart from the epibiotic subject of this research). Originally found in marine ecosystems, the *Bdellovibrios* have since been isolated from nearly all types of habitats, demonstrating a particular affinity for predation in the rich environs of biofilms, a possible shared predilection with *V. chlorellavorus* (Williams et al., 1995). All BALOs have been found to be obligate parasites, requiring the presence of a host cell in order to complete their lytic life cycle (Jurkevitch and Davidov, 2006). Several authors have reported a positive correlation between seasonal temperatures and recovered densities of BALOs from both the Chesapeake bay area and Australia (Sutton and Besant, 1994; Williams et al., 1982). Evidence of the same seasonal trend in the prey population of a particular genus opens the possibility that food source is driving the fluctuations in bacterial populations (Colwell et al., 1977). Whatever the precise cause, this agrees with observations of increased pathogenicity of *V. chlorellavorus* under warmer temperatures in cultures at the University of Arizona.
While the specifics of BALO predation differ from *V. chlorellavorus*, in the search for information on the dynamics of bacterial predators they can be informative. One study on the kinetics of the predatory interaction of *Bdellovibrio bacteriovorus* with *Escherichia coli* first made the observation that at high enough concentrations the normally periplasmic invader will engage in “bacteriophage-induced ‘lysis from without’” and went on to report experiments describing the interaction in great detail (Varon and Shil, 1968). They demonstrated the cycle of growth and crash of the predator population occurred over a 90 hour period under normal conditions with an inoculation ratio (IR) of 1:10 parasite:prey and that the proportion of the parasites that were attached to host cells decreased with increasing IR. Upon saturation of attachment to host cells, the addition of more *E. coli* did not cause the attachment of more *B. bacteriovorus* cells, while the addition of parasite cells did dramatically add to newly attached cells, which may inform management of *V. chlorellavorus* in algal culture if a similar dynamic exists. Recent reviews on the subject confess to a nearly complete lack of evidence for the mechanism of either attachment or periplasmic invasion by BALOs, with only inferred function of putative components of a type IV pilus system. For instance, *pilA* mutant BALOs lack the main retractable fiber of the system and thus are incapable of predation (implicating the pili in mode of action) (Sockett, 2009).

1.1.6 *Vampirovibrio chlorellavorus*: lifecycle of a microalgae predator

The first description of *V. chlorellavorus* in the literature was made on samples of microalgae cells observed to be degrading in a Ukrainian freshwater reservoir. Electron microscopy indicated the presence of a Gram-negative bacterium associating itself closely with the cell wall of the chlorophyte, *Chlorella vulgaris*, leading the authors to designate it *Bdellovibrio chlorellavorous* based on similarity to the genus of predatory prokaryotes (Gromov and
Mamkaeva, 1972). Shortly thereafter, a leading research group in the field of *Bdellovibrio* study published more details on the epibiotic attachment of the bacteria via a ‘pad’ like structure leading to the dissolution of internal algal cell contents in a matter of days. The group also noted that the initial designation of the bacterium was not appropriate, as all other *Bdellovibrios* prey upon Gram-negative bacteria by integrating themselves into the periplasmic space (Coder and Starr, 1978). While taxonomic assignment to the Deltaproteobacteria was retained, the organism was renamed *Vampirovibrio chlorellavorus* to reflect its unique life style (Gromov and Mamkaeva, 1980). Subsequent phylogenetic analysis based on the concatenated alignment of 109 single copy genes has placed *V. chlorellavorus* into the cyanobacteria phylum, establishing Vampirovibionales as a new order within the class Melainabacteria (Soo et al., 2014, 2015). The proposed life cycle of *V. chlorellavorous* was built initially upon microscopy observation and later elaborated by functional annotation of whole genome DNA sequencing. Pleomorphic life style was first inferred from electron micrographs demonstrating not only coccoid shaped cells attached to algal prey, but also smaller vibrioid shaped cells motile by the “clumsy” use of a single unsheathed flagella (Coder and Starr, 1978; Gromov and Mamkaeva, 1972). The sequencing and assembly of the bacterium’s 3.03 Mbp complete genome indicated 51.4% GC content while predicted gene functions allowed for more detailed predictions of the pathogenic life cycle. The authors proposed a five stage scheme as follows: first; prey location is driven by a flagellum and chemotaxis via the CheA-CheY signal pathway, second; attachment and conjugation to host cells is characterized by a full suite of type IV secretion system genes and predicted T-strand encoded enzymes, third; 108 protease genes and 123 carbohydrate-active enzyme genes predict the ingestion phase, fourth; binary fission of pathogen cells as indicated by the tubulin-like FtsZ, and finally; release back into the motile attack phase (Soo et al., 2015).
The host range of *V. chlorellavorous* has been shown to include exclusively *Chlorella spp.* with one study finding algal cell death resulting in all 31 tested *C. vulgaris, C. sorokiniana,* and *C. kessleri* strains, while strains of nine other species in the genus survived inoculation with the bacteria (Coder and Goff, 1986).


Ponchel, F., Toomes, C., Bransfield, K., Leong, F.T., Douglas, S.H., Field, S.L., Bell, S.M.,
fluorescence: An alternative to the TaqMan assay for a relative quantification of gene


Detection, Identification, and Quantification of Phytoplanktonic Fungal Parasites. Appl. Environ.
Microbiol. 75, 2545–2553.


Department of Energy’s aquatic species program: biodiesel from algae (National Renewable
Energy Laboratory Golden).

Singh, A., Nigam, P.S., and Murphy, J.D. (2011). Renewable fuels from algae: An answer to

Singh, S., Kate, B.N., and Banerjee, U.C. (2005). Bioactive compounds from cyanobacteria and


APPENDIX A:

DEVELOPMENT OF A REAL-TIME PCR METHOD FOR IDENTIFICATION AND QUANTIFICATION OF THE CHLORELLA SPP. BACTERIAL PATHOGEN, VAMPIROVIBRIO CHLORELLAVORUS

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ABSTRACT

The pathogenic bacteria, *Vampirovibrio chlorellavorus*, has been linked to profoundly adverse effects on the growth of *Chlorella sorokiniana* microalgae in mass culture systems intended for biomass production for biodiesel feedstock at testbed sites in the Southwest region of the United States. In an effort to protect this desirable crop species, a quantitative PCR assay was developed to target the bacterial 16S rDNA gene and multiplexed with an internal control 18S rDNA algal host gene target to inform management strategies in open outdoor growth ponds. The assay was implemented in field studies comparing growth of the microalgae and accumulation of the pathogen subjected to treatment of the cultures by the biocide, benzalkonium chloride. These experiments support the validity of the method and indicated an increase in culture longevity between biocide treated and untreated outdoor cultures (average of 21 and 11.3 days respectively) as well as decreased normalized disease severity calculations for treated and untreated cultures.
INTRODUCTION

The green micro-algae comprise a diverse group of single celled organisms capable of photosynthetic carbon fixation without the structural complexity of land plants. These miniature bio-refineries have become an attractive target for alternative energy production as they are capable of rapid proliferation, accumulation of as much as 80% of their biomass as lipids, and can be cultivated on marginal land (Chisti, 2008; Hu et al., 2008). While open pond systems have been shown to be the most economically feasible platform for algal biomass production (Jorquera et al., 2010), they present a substantial challenge in their susceptibility to invasion by a myriad of pests and predators (Carney and Lane, 2014). The University of Arizona collaborates with three other research groups to operate open systems to test algal culture productivity in the arid Southwest region of the United States. A field isolate of *Chlorella sorokiniana* (DOE 1412) was screened and selected for maximum specific growth rate (5.9 day\(^{-1}\)) under high temperature conditions (36\(^{\circ}\)C) similar to those experienced during the summer months (Huesemann et al., 2016).

Beginning in April 2014, phycosphere monitoring was routinely carried out on RAFT project cultures by analysis of ribosomal RNA small sub unit (rRNA SSU) gene sequences, which uncovered the presence of a known algal pathogen, *Vampirovibrio chlorellavorus* (Park et al., unpublished). This obligate parasite was first described as causing the discoloration and clumping of *Chlorella vulgaris* cells, as well as their dimorphic vibrioid and coccoid forms ranging in diameter from 0.3-0.6 \(\mu\)m, respectively (Gromov and Mamkaeva, 1972). Subsequent research by electron microscopy and genomic analysis place the intriguing organism in the cyanobacteria phylum, and demonstrate the attachment of bacteria to the exterior cell wall of *Chlorella spp.* cells, likely via a type IV secretion system, leading to the dissolution of internal
algal cell contents and collapse of the culture (Coder and Starr, 1978; Soo et al., 2015). It has been shown to shift from a motile free-living stage into the larger attached form and affect a relatively narrow host range of *Chlorella spps.* (Coder and Goff, 1986).

Effective methods for the detection and quantification of *V. chlorellavorus* are necessary both for monitoring production cultures, as well as laboratory experiments on mitigation techniques. Light microscopy observation is convoluted by variable cell morphology and minute size, particularly in mixed cultures where differentiation from other small bacteria frequently leads to misidentification. The predatory bacterium has also proven resistant to various attempts to culturing on agar media (Coder and Starr, 1978; Soo et al., 2015), joining a growing list of microorganisms that cannot be assessed by plate counts (Stewart, 2012). To address these limitations, molecular biologists have turned to techniques including fluorescence in situ hybridization (FISH) (Harmsen et al., 2000; Malic et al., 2009), microarray (Wilson et al., 2002), end point PCR (Matsuki et al., 1999), and quantitative real-time PCR (qPCR) (Rinttila et al., 2004).

Compared to conventional PCR, qPCR offers similarly high sensitivity and specificity, while also facilitating accurate quantification of the resultant PCR product. This study adopts a modified absolute quantification approach similar to recent experiments in the detection of microalgae species causing harmful algal blooms (Yuan et al., 2012; Zhang et al., 2015), designing a probe based qPCR assay targeting sequences encoding ribosomal RNA small subunits (SSU). To this end, the *V. chlorellavorus* 16S SSU and *C. sorokiniana* SSU genomic regions were amplified, sequenced, and used to design specific primer and probe sets for each target. Purified vectors containing the 16S and 18S fragments were used to generate standard curves relating quantification cycle (*C*ₙ) to measured gene copy number. The assays were then
tested in a multiplex reaction, allowing for the quantification of pathogen DNA in relation to the algal host regardless of variations between DNA extraction efficiencies. In order to assess the two distinct life phases of *V. chlorellavorus*, live culture samples were centrifuged and filtered to separate fractions containing the attached and free living bacterial cells respectively. The concentration of bacterial cells in each fraction was then quantified by application of the qPCR assay.

Finally, the qPCR assay was implemented to assess the efficacy of a chemical treatment for the control of *V. chlorellavorus* accumulation and pathogenicity on outdoor DOE 1412 monoculture. The quaternary ammonium complex, benzalkonium chloride, has been shown to be an effective biocide against a variety of bacterial cells (Houari and Di Martino, 2007; Jean et al., 1999). The recognized mode of antimicrobial action is that the amphiphilic character of the molecule allows for ingestion into, and disruption of, unprotected phospholipid membranes (Paulson, 2002). These experiments, in addition to confirmation of algal attached bacteria by scanning electron microscopy, establish an accurate, specific, and rapid tool for protection of *C. sorokiniana* cultures.

**METHODS**

*Strain sourcing and culturing*

The *C. sorokiniana* strain DOE1412 (also designated NAABB 2412) was originally isolated from surface water in Texas by Dr. Juergen Polle of Brooklyn College (Huesemann et al., 2013) and cultures were maintained on BG-11 media containing 17.6mM of NaNO₃, 0.22mM of K₂HPO₄, 0.03mM of MgSO₄·7H₂O, 0.2mM of CaCl₂·2H₂O, 0.03mM of citric acid·H₂O, 0.02mM of ammonium ferric citrate, 0.002mM of Na₂EDTA·2H₂O, 0.18mM of Na₂CO₃ and
BG-11 trace metal solution (Rippka and Herdman, 1992). Continuous lines were kept on petri plates of solid BG-11 containing 30 g/L of agar. Outdoor growth and select laboratory experiments were inoculated into a modified medium developed for high yield with minimal nutrient input in Pecos, Texas (PE07). The PE07 media contains 1.7mM of Urea ((NH$_2$)$_2$CO), 0.05mM of MgSO$_4$·7H$_2$O, 0.3mM of NH$_4$H$_2$PO$_4$, 1.4mM of Potash (KCl), 0.03mM of FeCl and BG-11 trace metal solution. Field experiments were conducted at the University of Arizona test site (+32° 16' 49.29", -110° 56' 9.82") in reactors of increasing scale providing sufficient inoculum for three open raceway systems including two 762 L traditional paddlewheels (PW) (Crowe et al., 2012) and a 5600 L Aquaculture Raceway Integrated Design (ARID) system (Waller et al., 2012).

**Scanning Electron Microscope (SEM) analysis**

Scanning electron microscopy (SEM) was carried out at University Spectroscopy & Imaging Facilities (USIF) at University of Arizona. Clean glass slides were prepared by coating with poll-L-lysine to promote cell adherence prior to being spotted with 200 μL of live algal cell culture. Equal volume of fixative (4% paraformaldehyde::2% glutaraldehyde (v/v) in 0.2M Sodium Phosphate Buffer pH 7.4) was then added to each sample before being triple rinsed in glass deionized water and successively dehydrated by increasing concentration of ethanol. Finally, the specimens were mounted and sputter coated (Hummer 6 Sputtering Device) before being imaged using a Hitachi S-4800 Type II / Thermo NORAN NSS EDS: Field-Emission scanning electron microscope.

**Total genomic DNA extraction culture fractionation**
Total genomic DNA was extracted from samples using a cetyltrimethylammonium bromide (CTAB) method modified from previous studies (Phillips et al., 2001). Samples were collected in 50 mL tubes from turbulent sections of outdoor reactors and biomass was harvested by centrifugation at 5000 rpm for 5 minutes. After decanting the supernatant, 20 mg of 1.4 mm stainless steel beads were added along with 1 ml CTAB buffer containing 20 μL β-mercaptoethanol. Algal cell walls were disrupted using a Mini-Beadbeater-96 (Biospec products Inc, Bartlesville, OK), followed by transfer of the homogenate to a sterile microfuge tube where an equal volume of chloroform: isoamyl alcohol (24:1) was mixed in by inversion. The mixture was centrifuged for 10 minutes at 9000 rpm, then the aqueous supernatant was transferred to a final microfuge tube and DNA was precipitated by 2/3 volume of cold isopropanol. After allowing overnight (16 hr) incubation at -20°C, DNA was sedimented by centrifugation for 10 minutes at 9000 rpm, supernatant was discarded, and the pellet was washed with 70% ethanol. Finally, the ethanol was removed and the pellet was dried before being resuspended in 20 μL Tris-HCl buffer (TE, pH 7.2).

The presence of *V. chlorellavorus* suspended in culture media versus cells associated with the algal cell biomass was assessed by the fractionation of liquid samples. Algal cells and associated microbes were harvested by centrifugation of 1 mL samples at 7000g for 5 mins, and the resulting supernatant (suspended cells) was transferred into sterile 1.7 mL microfuge tubes by pipette. The biomass pellet was then processed for isolation of genomic DNA as described above beginning at the cell wall disruption (bead beating) step. Concurrently, material in the supernatant media was concentrated by vacuum filtration through sterile 0.2 μm pore sized Whatman polycarbonate membranes (GE Healthcare, UK). The bacteria loaded filters were then moved into sterile tubes where they were bathed in warm (60 °C) CTAB buffer for 5 mins before
15 mins incubation in a 65 °C heating block to lyse prokaryote cells. Resulting cell lysate was extracted for genomic DNA following the method above beginning at the chloroform: isoamyl alcohol separation step.

**Sequencing of C. sorokiniana and V. chlorellavorus SSU rDNA**

Genomic DNA was isolated from algal biomass samples collected from laboratory DOE 1412 monoculture and open growth reactors at the University of Arizona field site by the method described above and utilized as template for conventional PCR amplification of both algal and bacterial 18S and 16S SSU rDNA respectively. Near full length SSU sequences were amplified by universal 16S primers F16SW and R16SW (Weisburg et al., 1991), or previously designed algal 18S primers F18S and R18S (Park et al., unpublished) (Table 1). The resulting 16S (~1.5 kb) and 18S (~1.25 kb) PCR fragments were ligated into pGEM-Teasy vector (Promega Inc, WI, USA) then transformed into *Escherichia coli* DH5α competent cells. Successfully transformed colonies were selected by blue white screening on agar plates containing X-gal and ampicillin and then used as template for PCR of the inserted DNA fragments. PCR products were then sent to the University of Arizona Genetics Core (UAGC) and sequenced on a 3730 DNA Analyzer (Applied Biosystems, CA, USA). Sequences were assessed by BLAST alignment against the NCBI non redundant database and have been submitted to GenBank for both DOE 1412 (accession no. KM068038) and *V. chlorellavorus* (accession no. KP710184).

**qPCR assay design**

The sequences described in “**Sequencing of C. sorokiniana and V. chlorellavorus SSU rDNA**” were used as templates for the design of the primers and hydrolysis probe sets for each target organism. Putative sets were designed using the PrimerQuest® Tool (Integrated DNA
Technologies, USA), and were manually screened based on established guidelines for amplicon size, GC content, and secondary structure (Taylor et al., 2010). Secondary structure and other primer parameters were checked by use of the primer3 algorithm (Rozen and Skaletsky, 1999). The specificity of *V. chlorellavorus* 16S primers/probe was assessed by both the Ribosomal Database Project (RDP) Probe Match tool (Cole et al., 2005) and the SILVA TestPrime 1.0 tool (Klindworth et al., 2013). An alignment of closely related green algae species was used to determine the specificity of the *C. sorokiniana* primers and probe.

**qPCR**

Assays were performed using a StepOnePlus™ Real Time PCR system (Applied Biosystems, USA) where reactions were arranged in 96 well plates. Both bacterial and algal assays were multiplexed into single 25 μL reactions containing 12.5 μL TaqMan® Universal PCR Master Mix, 1.25 μL of each qVV_F/R and D18S_F/R primer pair (10 μM), 0.625 μL of qVV_P and D18S_P probes, and 7.75 μL of nuclease free water. The cycling parameters were executed as follows: 50 °C for 2 mins, 95 °C for 10 mins, 40 cycles at 95 °C for 10 s, and a final step of 58.8 °C for 60 s. All reactions were carried out in triplicate, with each PCR run including a standard curve. Limits of detection (LOD), blank (LOB), and quantification (LOQ) were calculated using the following natural log modified equations respectively; LOD = e^{-\frac{B_{SC}}{M_{SC}}}, LOB = mean_{blank} - (1.645 \times SD_{blank}), LOQ = mean_{LOB} + (3 \times SD_{LOB}) where \(B_{SC}\) and \(M_{SC}\) indicate the intercept and slope of the standard curve and SD indicates standard deviation (Armbruster and Pry, 2008).

**Construction of *C. sorokiniana* and *V. chlorellavorus* qPCR standard curves**

Vectors (pGEM-Teasy) containing the nearly full-length 16S and 18S rDNA fragments described above were utilized as template for conventional PCR to amplify the short qPCR target...
fragments. The primer sets qVV_F/qVV_R and D18S_F/D18S_R yielded small fragments of *V. chlorellavorus* (113 bp) and DOE 1412 (119 bp) DNA respectively, that were subsequently ligated into pGEM-Teasy. Following the transformation of DH5α cells, colonies were grown overnight (16hr) in LB media and plasmid was purified from cell cultures using geneJET plasmid miniprep Kits (Thermo scientific, USA). Because previous studies indicate the over estimation of copy number with circular plasmid standards (Hou et al., 2010), each plasmid was linearized by enzyme digestion with PstI (New England Biolabs, USA) before quantification by spectrophotometer (NannoDrop 2000, Thermo scientific, USA). The plasmid copy number concentration (NCP; copies μL⁻¹) was calculated as follows: NCP = (PC × 10⁻⁹ × 6.02 × 10²³) ÷ (N × 660), where PC represents the concentration of plasmid (ng μL⁻¹) and N represents the nucleotide base number of recombinant plasmids. Deionized water was used to make series of tenfold dilutions for each plasmid to use as template in standard curve experiments.

*C. sorokiniana SSU rDNA copy number determination*

High cell density sample was taken from a monoculture of DOE 1412 during the exponential growth phase. Deionized water was used to make a series of six fivefold dilutions of the initial material, with cells being homogenized by aspiration. The density of each dilution sample was measured by light absorbance at 750 nm wavelength on a spectrophotometer (Spectronic BioMate3, Thermo sci, USA) as well as cell counts under both bright-field (BR) and fluorescent (FL) illumination using an automated imaging system (Cellometer® Vision, Nexcelom Biosciences, USA). Triplicate samples were taken from each dilution and DNA was isolated in parallel as described in “Total genomic DNA extraction and culture fractionation” and assayed in triplicate by the method outlined in section, “qPCR”. The number of 18S copies per algal cell
was estimated by plotting the calculated copy number (according to standard curves) as a function of BR cell counts.

Field study of V. chlorellavorus control strategy by benzalkonium chloride treatment

In order to compare the effects on benzalkonium chloride (BAC) on outdoor cultures, experiments were conducted in two identical and collocated PW oval shaped reactors (PW1 and PW2). Each was operated according to semi-continuous biomass production protocols as follows. Reactors were inoculated with laboratory grown DOE 1412 cells to an optical density of approximately 0.2 (OD750), equivalent of between 3·10^6 and 5·10^6 cells/ml, in PE07 media (described in Strain Sourcing and Culturing). Collected biomass was maximized by harvesting 75% of culture volume during exponential algal growth (OD750 ≥ 1.5), followed immediately by replenishment of water and 1X equivalent media nutrients. Cultures were considered crashed upon observed decrease in algal cell density over two consecutive days. One PW unit was treated with a dose of 2 ppm benzalkonium chloride every fourth day during growth with the other left as an untreated control. Drop samples were processed from each reactor every two days for qPCR assessment of V. chlorellavorus as described above. Biomass productivity was assessed by ash free dry weight (AFDW) measurements used for the calculation of areal productivity similarly to previous studies, wherein the change in g/L concentration was subtracted from the previous measure, multiplied by the volume of the reactor, and divided by the surface area and time (Pedroni et al., 2004). In order to reduce bias against the declining biomass in the final days of untreated cultures, average productivity was based only on exponential growth phases of each run. Calculations of disease severity were made by finding the relative area under disease curve stairs (rAUDPS) algorithm (Simko and Piepho, 2012), an improved method for quantifying the area under a curve defined by the accumulation of V.
chlorellavorus rDNA (16S:18S ratio) over time as implemented in the agricolae package in R (De Mendiburu, 2014; R Core Team, 2013).

RESULTS

Visualization of putative V. chlorellavorus cells

In addition to molecular identification of V. chlorellavorus cells in DOE 1412 culture, the presence of the bacteria was observed by scanning electron microscopy. The micrographs generated from sample collected from outdoor monoculture indicate the presence of bacterial cells with diameters between ~0.8 and 1.2 μm in close association with the larger algal cells (Fig. 1). The higher magnification images reveal an intricate ultrastructure of fine hair like protrusions covering the bacterial cells, and both intact and lysed algal cells are visible in each image.

qPCR primer-probe specificity

The gene encoding 16s rRNA has proven extremely useful for culture-independent identification of prokaryotes in part because of its high degree of conservation across all known species (Amann et al., 1995). This characteristic also necessitates the careful selection of primer and probe annealing sites in more variable regions of the sequences (Marchesi et al., 1998). Therefore, the design of the qVV_F/R and qVV_P probes was evaluated for specificity against the SILVA 16S rRNA database for accessions that matched all three oligonucleotide sequences (Table 2). The entire non-redundant database consists of 576,050 unique 16S sequences, of which qVV_F/R and qVV_P matched 184 and 135 accessions respectively. Among the sequences matching both analyzed sets, 19 were classified in the Vampirovibrionales family,
which contains *V. chlorellavorus* (Soo et al., 2014). Only one accession was shared within each of the Firmicute and Proteobacteria phyla, indicating a high degree of specificity.

The goal for the design of primers and probes targeting the 18S rRNA gene was to provide internal normalization of *V. chlorellavorus* quantification for each sample against the amount of extracted algal DNA. The D18S_F/R and D18S_P sequences are homologous to *C. sorokiniana* 18S rDNA, as well as several other chlorophytic green microalgae species. Previous analysis of 18S rDNA in both the University of Arizona field ponds, and nearby environmental samples, indicated a number of abundant eukaryotic organisms (Park et al., unpublished) that were included in a multiple sequence alignment to indicate sequence homology within the targeted regions (Fig. 2). In the context of the high cell density monocultures grown for biofuel production, there is a low probability of amplification of 18S rDNA from off target eukaryotes.

**Standard curve**

Standard curves for both qPCR target DNA fragments were established by finding the linear relationship between their respective C\textsubscript{q} values and the denary logarithms of plasmid copy numbers (Fig. 3). Both curves represent tenfold dilution series that span a wide range of plasmid concentrations between $10^4$ and $10^{11}$ copies per μL used as template. The linear regression function in base RStudio yielded the following equations for the *V. chlorellavorus* 16S and *C. sorokiniana* 18S assays respectively: $y = -4.1x + 50, r^2 = 0.997$ and $y = -3.9x + 57, r^2 = 0.992$. These equations allow for the interpolation of DNA copy number from all subsequent unknown experimental samples based on their measured C\textsubscript{q} readings. The sensitivity of each assay was determined by calculating LOD, LOB, and LOQ based on repeated nuclease free water blank reactions ($n=40$), indicating that the bacterial rDNA can be reliably detected (95% confidence interval) above 19 copies, and algal rDNA above 131 copies (Table. 3).
Determination of 18S rDNA copy number

Previous investigation has indicated a very wide range of 18S SSU rDNA copy number encoded in picoeukaryote genomes, from a single copy to 12,000 in the large dinoflagellate, *Akashiwo sanguinea* (Zhu et al., 2005). The relationship between 18S gene copy number and DOE 1412 cell count numbers was assessed across relevant algal culture densities between $10^4$ and $10^8$ cells/mL (Fig. 4). Linear regression of the data is described by the well fit equation, $y = 313x - 1e^9, r^2 = 0.996$. The slope of the equation describes the number of gene copies per counted algal cell, indicating over 300 encoded in each genome. The Zhu et al. 2005 study also estimated a linear correlation between 18S copy number and cell size, indicating that the two share a positive relationship. The 18S copy number estimated in the current study for the 5-7 μm diameter DOE 1412 isolate agrees closely with the ~7 μm green algae chlorophyte, *Chlamydomonas concordia*, estimated to contain between 300 and 400 copies. This result allowed for the refinement of the qPCR assay by relating *V. chlorellavorus* 16S rDNA copy number measurements to algal cell number, thereby defining the vampiro disease ratio (VDR) for each measurement by the equation; $VDR = \frac{16S\ rDNA\ copy\ #}{18S\ rDNA\ copy\ #} \times 313$. Results of the same 18S copy number experiment on samples of DOE1412 field cultures yielded a very similar relationship, indicating 303 copies per algal cell (Fig. 5).

Biocide treatment effects on outdoor DOE 1412 biomass production

Initial implementation of the *V. chlorellavorus* qPCR assay was conducted at the University of Arizona as part of a collaborative microalgae biomass production research project with the moniker of regional algal feedstock testbed (RAFT), at a test site with a previously reported prevalence of the pathogenic bacteria (Park et al., unpublished). The experiment centered on the
collection and assessment of samples taken in time course from three independent growth cycles of DOE1412 culture in two collocated paddlewheel (PW) reactors. Both units were cultivated under the same standardized procedures with one being chemically treated by regular dosages of 2 ppm benzalkonium chloride (BAC) and the other left as a no treatment control (NT). The data from these assays indicated not only that the pathogen can be detected and quantified through the course of the runs, but also a suppressing effect on its accumulation in the BAC treated reactors (Fig. 6, 7, 8). The most pronounced observation amongst this data is the increased longevity of BAC treated cultures, as indicated by an average growth duration of 21 days compared to the average growth observed in NT reactors of 11.33. This increase in active growing days results in higher total productivity in each treated reactor against its paired control (55.5% to 61.2% increased production), despite a decrease in average biomass productivity during the exponential growth phase of runs 18, 23, and 25 (16.6%, 48.8%, and 6.5% reductions respectively) due to non-specific biocide effects on the microalgae from BAC treatment (Table. 4).

In order to assess the overall disease severity on each microalgae cultivation cycle, the area under the curve formed by the plotting of qPCR derived VDR over time was estimated using the plant epidemiology calculation for relative area under disease progress stairs (rAUDPS). This allowed for estimates under irregular disease curves without a well fit model, and was subsequently normalized to each run by dividing by growth duration, as in previous research (Fry, 1978). These calculations revealed very large inter-run differences in disease severity, indicating that the NT experienced between one to as much as seven orders of magnitude greater severity than their paired BAC treated counterparts (Table 5). Despite being operated under variable environmental parameters experience throughout the summer and fall months, the average rAUDPS calculation was far higher in the NT reactors (83.2) compared to BAC treated
(0.00107), though high variance prevents statistical significance as computed by a paired T test analysis.

DISCUSSION

The quantitative assay for the assessment of *V. chlorellavorus* infection of *C. sorokiniana* microalgae culture developed here promises to be an invaluable tool in the effort to inform mitigation of the pathogenic effects in open outdoor reactor units. The detection sensitivity offered by qPCR is not easily surpassed, and if implemented with the proper work pipeline could yield results on the same day of sampling. This would allow for near real time notification of an infection event and allow for a decision to enact some form of treatment on the affected reactor(s) and/or choose to harvest cultures early to save salvage precious algal biomass (McBride et al., 2014). The inclusion of the qPCR target *C. sorokiniana* helps greatly to ensure not only that the replicability of results is high (by reducing reliance on extremely consistent DNA isolation between samples), but also allows for a good metric for the assessment of disease severity by providing a quantitative measure with respect to the host. Plant epidemiologists have stressed the importance of the use of an accurate and unbiased technique after demonstrating the lack of predictive power in less stringent methods (Lindow, 1983).

The application of the assay has already proven useful in the assessment of use of the biocide, benzalkonium chloride (BAC), giving strong indications of its effectiveness against the disease. However, further field assessments will be needed to validate the result, as well as to determine whether the negative impact on microalgae growth can hold up to economic necessities of high productivity for the application as a biofuel feed stock (Amer et al., 2011). Nevertheless, informative trends arise when comparing the three independent *C. sorokiniana* growth cycles. For instance, previous evidence has indicated that rainfall leads to loss of productivity and death
of C. sorokiniana. During this study, the highest recorded titer of V. chlorellavorus in the raceways (2.56x10^6 VDR) was immediately preceded by the most substantial rainfall (42.7 L added) into the open reactors throughout experimental timeframes (Fig. 7). This phenomenon may be caused by deposition of additional V. chlorellavorus cells, which are small enough to be carried into the atmosphere as is the case for many other bacteria (Burrows et al., 2009). Host density relative to pathogen presence may be an important indicator of impending pathogenicity, as has been observed for predatory Bdellovibrio spp., which share many characteristics with V. chlorellavorus (Varon and Shil, 1968). None of the initial detection events in any of the reactors occurred prior to the culture reaching a density of at least 1.0 (OD 750), but this relationship cannot be disentangled from the possibility of a latency period, as each initial observation occurs at between 7 to 9 days after the culture start (Figs 4,5,6). The accuracy of this assay should also be leveraged in laboratory experimentation on mechanisms of the predatory habits of the V. chlorellavorus for the purpose of scientific inquiry into this curious organism, in addition to identifying vulnerabilities that may be targeted by more precise methods of control than the general biocide utilized in the experiments presented here.

ACKNOWLEDGMENTS

A great deal of thanks is required for the many people without whom this work would have never been completed. C.C. Brown and N. Kitchen deserve high praises for their superb contributions to culture purity, production, and assay design. Many thanks to C. Galves and S. Lee for their tireless assistance in the laboratory, and to all the members of the ARID raceway team. None of this would have even begun without the support and mentoring of Dr. K. Ogden and the generous patience of Dr. J.K Brown.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Scanning electron micrograph of clumped DOE 1412 cells infected with *V. chlorellavorus* at A) 5,000X magnification and B) artificially-colored *V. chlorellavorus* (yellow) infected DOE1412 (green) at 15,000X magnification.

**Figure 2.** Alignment of the partial 18S rDNA sequence of seven microalgal strains, and 13 eukaryotic organisms identified in the field site open pond reactors and the surrounding area. The *C. sorokiniana* (DOE 1412) primers and probe D18S_F, D18S_R, and D18S_P complementation sites are indicated by the black forward arrow, reverse arrow, and bar respectively.

**Figure 3.** Results of replicate standard curve experiments displayed as the cycle threshold (Ct) response to known numbers of target bearing plasmids for both the A) *C. sorokiniana* 18S sequence and B) *V. chlorellavorus* 16S sequence. Plasmid copy numbers are represented in log base 10. Error bars indicate ± standard deviation n=5.

**Figure 4.** Relationship between laboratory *C. sorokiniana* 18S rDNA copy number as calculated by the standard curve equation and the measured cell count number of diluted algae monoculture. The linear regression line is shown in blue and the corresponding equation is indicated along with the $r^2$ goodness of fit. Error bars represent ± standard deviation of biological replicates n=3.

**Figure 5.** Relationship between field *C. sorokiniana* 18S rDNA copy number as calculated by the standard curve equation and the measured cell count number of diluted algae monoculture. The linear regression line is shown in blue and the corresponding equation is indicated along with the $r^2$ goodness of fit. Error bars represent ± standard deviation of biological replicates n=3.
Figure 6. Benzalkonium chloride (BAC) effects on DOE 1412 growth and *V. chlorellavorus* accumulation in field reactors. The panels summarize data from an algal growth cycle between May, 19 and Jun, 9, 2016 with A) culture temperature is displayed as recorded by continuous data logger from PW1 reactor unit and compared across a time course of 25 days of growth against B) the optical density (absorbance at 750 nm) of *C. sorokiniana* culture in collocated PW reactor units either treated with BAC or a no treatment control (NT) as well as rainfall events quantified by the volume of water added to reactors. C) *V. chlorellavorus* accumulation is displayed in both biomass (B) and media (M) fractions by the ratio of 16S rDNA per algal cell 18S rDNA detected on a logarithmic scale which excludes initial time point samples of undetected 16S rDNA.

Figure 7. Benzalkonium chloride (BAC) effects on DOE 1412 growth and *V. chlorellavorus* accumulation in field reactors. The panels summarize data from an algal growth cycle between Aug, 30 and Sept, 22, 2016 with A) culture temperature is displayed as recorded by continuous data logger from PW1 reactor unit and compared across a time course of 25 days of growth against B) the optical density (absorbance at 750 nm) of *C. sorokiniana* culture in collocated PW reactor units either treated with BAC or a no treatment control (NT) as well as rainfall events quantified by the volume of water added to reactors. C) *V. chlorellavorus* accumulation is displayed in both biomass (B) and media (M) fractions by the ratio of 16S rDNA per algal cell 18S rDNA detected on a logarithmic scale which excludes initial time point samples of undetected 16S rDNA.

Figure 8. Benzalkonium chloride (BAC) effects on DOE 1412 growth and *V. chlorellavorus* accumulation in field reactors. The panels summarize data from an algal growth cycle between Sept, 23 and Oct, 12, 2016 with A) culture temperature is displayed as recorded by continuous
data logger from PW1 reactor unit and compared across a time course of 25 days of growth against B) the optical density (absorbance at 750 nm) of *C. sorokiniana* culture in collocated PW reactor units either treated with BAC or a no treatment control (NT) as well as rainfall events quantified by the volume of water added to reactors. C) *V. chlorellavorus* accumulation is displayed in both biomass (B) and media (M) fractions by the ratio of 16S rDNA per algal cell 18S rDNA detected on a logarithmic scale which excludes initial time point samples of undetected 16S rDNA.
### Tables

**Table 1** Summary of primers and probes used in this study

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$^a$Probe modifications indicated within sequences as FAM or JOEN dyes and ZEN or 3IABkFQ (Iowa Black® FQ) quenchers (Integrated DNA Technologies, IA, USA).
### Table 2 Summary of in silico analysis of *V. chlorellavorus* primers and probe specificity

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<sup>a</sup> SILVA taxonomic designations are displayed in descending rank, with those belonging to the same phylum separated by grey/white shading

<sup>b</sup> Indicates the number of sequences matching the oligonucleotides in each respective taxonomic rank

<sup>c</sup> Indicates the percentage of matches for each taxonomic rank
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<thead>
<tr>
<th>Target</th>
<th>Limit of Detection&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Limit of Blank&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Limit of Quantification&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. chlorellavorus</em></td>
<td>12.67</td>
<td>9.87</td>
<td>18.67</td>
</tr>
<tr>
<td><em>C. sorokiniana</em></td>
<td>97.68</td>
<td>56.34</td>
<td>130.56</td>
</tr>
</tbody>
</table>

<sup>a</sup> All limit calculations reported in units of target DNA copy number
Table 4 Algal biomass productivity of treated and untreated outdoor reactors

<table>
<thead>
<tr>
<th>RAFT Run&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reactor</th>
<th>Treatment</th>
<th>Average Exponential Productivity (g/m&lt;sup&gt;2&lt;/sup&gt;/day)</th>
<th>Duration (d)</th>
<th>Total Productivity (g/m&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>PW2</td>
<td>BAC</td>
<td>12.40</td>
<td>20</td>
<td>292.35</td>
</tr>
<tr>
<td>18</td>
<td>PW1</td>
<td>NT</td>
<td>14.46</td>
<td>13</td>
<td>187.97</td>
</tr>
<tr>
<td>23</td>
<td>PW1</td>
<td>BAC</td>
<td>7.86</td>
<td>24</td>
<td>188.68</td>
</tr>
<tr>
<td>23</td>
<td>PW2</td>
<td>NT</td>
<td>11.70</td>
<td>10</td>
<td>117.02</td>
</tr>
<tr>
<td>25</td>
<td>PW1</td>
<td>BAC</td>
<td>6.33</td>
<td>19</td>
<td>120.19</td>
</tr>
<tr>
<td>25</td>
<td>PW2</td>
<td>NT</td>
<td>6.74</td>
<td>11</td>
<td>74.09</td>
</tr>
</tbody>
</table>

<sup>a</sup> Matched RAFT run numbers indicate cultures grown during the same time period
### Table 5  Disease severity calculations of treated and untreated outdoor reactors

<table>
<thead>
<tr>
<th>RAFT Run</th>
<th>Reactor</th>
<th>Treatment</th>
<th>Biomass Normalized RelAUDPS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Media Normalized RelAUDPS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>PW2</td>
<td>BAC</td>
<td>3.22E-02</td>
<td>N/A</td>
</tr>
<tr>
<td>18</td>
<td>PW1</td>
<td>NT</td>
<td>1.01E-01</td>
<td>N/A</td>
</tr>
<tr>
<td>23</td>
<td>PW1</td>
<td>BAC</td>
<td>2.01E-05</td>
<td>1.62E-04</td>
</tr>
<tr>
<td>23</td>
<td>PW2</td>
<td>NT</td>
<td>2.49E+02</td>
<td>4.81E+01</td>
</tr>
<tr>
<td>25</td>
<td>PW1</td>
<td>BAC</td>
<td>3.14E-06</td>
<td>2.23E-04</td>
</tr>
<tr>
<td>25</td>
<td>PW2</td>
<td>NT</td>
<td>2.35E-04</td>
<td>3.89E-03</td>
</tr>
</tbody>
</table>

<sup>a</sup>Relative Area Under Disease Progress Stairs calculations are normalized by growth duration
Figures

Figure 1
Figure 2
Figure 3

A)  

\[ y = 57 - 3.0 \times x, \quad r^2 = 0.992 \]

B)  

\[ y = 50 - 4.1 \times x, \quad r^2 = 0.997 \]
Figure 4

\[ y = -1\times10^9 + 313 \cdot x, \quad r^2 = 0.996 \]
Figure 5

\[ y = -2.2 \times 10^9 + 303 \cdot x, \quad r^2 = 0.975 \]
Figure 7
Figure 8