Algal Research 41 (2019) 101535

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Evidence for induced allelopathy in an isolate of *Coelastrella* following co-culture with *Chlorella sorokiniana*

Alina A. Corcoran[a,1], Mark Seger[b,1,2], Rongli Niu[c,3], Nagamany Nirmalakhandan[c], Peter J. Lammers[b,2], F. Omar Holguin[d], Wiebke J. Boeing[e,*]

[a] New Mexico Consortium, Los Alamos, NM 87544, USA
[b] Energy Research Laboratory, New Mexico State University, Las Cruces, NM 88003, USA
[c] Department of Civil Engineering, New Mexico State University, Las Cruces, NM 88003, USA
[d] Department of Plant and Environmental Sciences, New Mexico State University, Las Cruces, NM 88003, USA
[e] Department of Fish, Wildlife and Conservation Ecology, New Mexico State University, Las Cruces, NM 88003, USA

ARTICLE INFO

Keywords:
Microalgae
Phytoplankton
Algae
Induced
Competition-induced
Competitor-induced
Chemical defense
Ecological cost
Chemical communication

ABSTRACT

Despite the potential of microalgae to meet growing societal demands for food, fuel, and nutraceuticals, the industrial economics of production systems are limited by low crop productivity and stability driven, in part, by invading pests such as competing algae. Effective management strategies to mitigate this challenge rely on a clear understanding of the interactions between microalgal production strains and invaders. Here, we take advantage of the invasion of *Chlorella sorokiniana* culture in the field by a wildtype organism later isolated and identified as a *Coelastrella* species to better understand the dynamics between these strains. In a set of experiments focused on invasibility, we found that coexistence of strains was possible, but that *Coelastrella* was the competitive dominant. To determine if this advantage was due to allelopathic interactions, we grew both strains in media that contained cell-free exudates from monocultures of *Coelastrella*, monocultures of *Chlorella*, or co-cultures of both strains. *Coelastrella* exudates did not inhibit the growth of *Chlorella*, but exudates from the co-culture of both strains did. *Chlorella* also exhibited some self-inhibition to its own exudate at high concentrations. This work demonstrates the potential for inducible allelopathic interactions between two phytoplankton strains. Future work focused on this phenomenon, through metabolomic and metagenomic lenses, would greatly contribute to our understanding of allelopathy in aquatic systems.

1. Introduction

Microalgal feedstocks have great potential to meet growing societal demands for food, fuel, and nutraceuticals. Yet, industrial scale production of microalgae is limited by technical and economic barriers, including the first order drivers of crop productivity and stability. Industrial producers typically cultivate microalgae as monocultures, using highly productive strains with consistent biomass composition. Moreover, to satisfy industrial economics, microalgae are commonly grown outdoors in open ponds [1–3] that are subject to invasions by predators, competitors, and parasites [4–6]. Each of these invaders can cause a sharp decline or "crash" of the algal crop. Invading algae are particularly problematic because they alter the biochemical composition of the crop, which can, in turn, affect downstream processing [7].

Strategies that aim to minimize invasion or reduce losses include the utilization of high inoculation densities, use of extreme culture conditions that are unfavorable to invasive species, and biological, chemical, and mechanical treatment [5,8–10]. Moreover, to develop effective treatment strategies at the industrial scale, it is often useful to develop biological model systems in the laboratory. Such model systems, often developed using organisms that cause crashes in the field, allow for tool development to track and proactively control invaders before they decimate crops [11,12]. Underpinning such model systems is the aim to understand species interactions. In this work, we take advantage of an invasion of a microalgal production strain in the field to develop

* Corresponding author at: 132 Knox Hall, New Mexico State University, Las Cruces, NM 88003, USA.
E-mail address: wboeing@nmsu.edu (W.J. Boeing).
[1] These authors contributed equally to this manuscript.
[2] Current address: School of Sustainable Engineering and the Built Environment, Arizona State University, Phoenix, AZ 85287, USA.
[3] Current address: School of Biomedical Sciences, Huaqiao University, 361021 Xiamen, China.

https://doi.org/10.1016/j.algal.2019.101535
Received 13 January 2019; Received in revised form 19 April 2019; Accepted 2 May 2019

laboratory experiments focused on species interactions – and more specially allelopathy.

Allelopathy has been broadly defined as the direct or indirect negative effect of one plant or microorganism on another, although beneficial interactions have also been considered allelopathic [13,14]. With respect to phytoplankton, the term allelopathy is specifically applied to the inhibitory effects of secondary metabolites produced by one species on the growth or physiological function of another species [15]. The exudation of secondary metabolites allows the producer of the allelopathic chemical(s) to monopolize limiting resources (e.g., nutrients, light) for its own population growth. The study of allelopathic effects in microalgae has a long history and recent work has matured to include quantification of the importance of allelopathy in natural systems as well as an understanding of chemical signaling mechanisms [16–20]. For example, it has been demonstrated both theoretically and empirically that allelopathic interactions prevent competitive exclusion and promote phytoplankton biodiversity in natural systems [21–23]. Conversely, it has also been shown that chemical signaling allows for the development and persistence of less diverse harmful algal bloom taxa [24–27]. Yet, classical plant defense theories remain inadequate to explain allelopathy in phytoplankton [28]. To advance this discipline, new approaches such as proteomics and coupling of chemical and molecular tools have helped to elucidate the complex chemical signaling mechanisms across phytoplankton that can affect community structure in the field [29–31]. In addition, from an applied perspective, researchers have recognized the potential of utilizing allelopathic compounds in algal production systems [32,33].

In this study, we focus on the allelopathic interactions between two microalgae: *Chlorella sorokiniana* and a *Coelastrella* species. Both *Chlorella* and *Coelastrella* are freshwater chlorophytes, with the former in the family Chlorellaceae and the latter in the family Scenedesmaceae. Strains of *Chlorella* are often used in algal production systems because of their lipid and carotenoid profiles, as well as physiological properties such as growth rates and modes of nutrition [34]. Some species of *Chlorella* are known to produce allelopathic chemicals [35,36], but there is no evidence in the literature that *Coelastrella* has this same ability. Here, we follow up on the invasion of a production strain of *Chlorella sorokiniana* by a wildtype isolate in the genus *Coelastrella*. We use this model system to quantify the potential for allelopathic interactions between these strains and generate foundational knowledge upon which additional studies can be built. Our work provides evidence that the competitive advantage of *Coelastrella* is conferred by the production of an allelopathic chemical.

2. Methods

2.1. Microalgae strain sources and characterization

Chlorella sorokiniana strain 1228, a clonal isolate derived from the *C. sorokiniana* UTEX 1230, was obtained from R. Sayre (Los Alamos National Laboratory, NM, USA). *Chlorella sorokiniana* was maintained photoautotrophically in BG11 media [37,38] enriched with 1% CO_2 in batch culture in the laboratory. Temperature was maintained between 22 and 24 °C and light was supplied continuously by cool white fluorescent bulbs (USHIO, Cypress, CA, USA) with Photosynthetically Active Radiation (PAR) at the surface of the flask of ~600 μmol $m^{-2} s^{-1}$.

The wildtype strain of *Coelastrella* was isolated from a field culture of *Chlorella sorokiniana*, which was being scaled in an enclosed outdoor raceway-style photobioreactor (PBR, see Section 2.2. "Outdoor Cultivation"). Specifically, we used repeated streaking onto BG11 plates to isolate single colonies for subsequent culturing [39]. The purity and identification of each culture was ensured morphologically by microscopy and molecularly by conventional PCR sequencing. The later was done using genomic DNA extracts (PowerSoil DNA isolation kit; MoBio, Carlsbad, CA) from 24 cultures derived from single colonies and universal primers that target the 18S rDNA and ribosomal ITS1 regions. Specificity and size of PCR amplicons were verified by gel electrophoresis. Subsequent sequencing of the amplicons was carried out bi-directionally using the same primers employed for PCR amplification (Functional Biosciences; Madison, WI). Sequence data were analyzed and assembled using Geneious® (V6.1.4; Biomatters Ltd., New Zealand) and the resulting consensus sequences were subjected to standard nucleotide similarity searches via BLASTn [40] against the NCBI non-redundant database using standard parameters to determine their identities. The 18S rDNA and ITS1 sequences generated in this study were deposited in the GenBank database (accession numbers: *Coelastrella* (KP167584 and KP167582, respectively) and *Chlorella* 1228 (KP167583 and KP167581, respectively).

2.2. Outdoor cultivation

On February 13, 2014, a single laboratory-scaled and DNA-verified 20-L carboy culture of *Chlorella sorokiniana* was inoculated into a single polyethylene-enclosed, raceway-style photobioreactor or PBR [41] with a total volume of 120 L and footprint of 1.2 m^2. The PBR was assembled within an unheated agricultural hoop-house at the Fabian Garcia Science Center at New Mexico State University (Las Cruces, NM). The PBR was filled with BG-11 media prepared in a 400-L mixing tank with Las Cruces municipal city water. This water was characterized by a specific conductance of ~600 uS cm^{-1}, hardness of ~200 mg $CaCO_3$, and a pH of 8.0. Nitrate and orthophosphate were not detectable and ~1.0 mg L^{-1} respectively and trace nutrients were minimal. The field medium contained $NaNO_3$ at half-strength (i.e., N concentrations reduced by half to 8.8 mM) compared to lab medium. After inoculation, the culture was maintained at 10 cm depth with water circulation via paddlewheel at a rate of 20–25 cm s^{-1}. Air was bubbled twenty-four hours a day, and the culture was enriched with CO_2 to 1% (vol/vol) between the hours of 7:00 a.m. and 5:00 p.m. each day. Maximum daily PAR in the hoop-house ranged from 750 to 850 μmol photons $m^{-2} s^{-1}$ during the growth period. No artificial light was provided.

To monitor culture health and identify pests, we used a Benchtop B3 Series FlowCam (Fluid Imaging Technology, Yarmouth, ME, USA). Following previous established protocols for this site [42], we enumerated and measured at least 10,000 organisms per sample. We created strain-specific sorting libraries based on size, shape, and fluorescence using training samples and then employed these libraries on our field data sets to distinguish between and enumerate *Chlorella* and *Coelastrella*.

2.3. Laboratory experiments

Two sets of experiments were conducted: one to assess the invasibility of *Chlorella sorokiniana* by the wildtype *Coelastrella* isolate and a second to determine if the species interactions were due to a release of exudates by the cells in culture.

2.3.1. Invasibility experiments

In the first set of experiments, we cultured each of the strains alone (monoculture controls) and in co-culture (co-culture treatments) at three different starting ratios based on cell abundance: (1) 90% *Chlorella*/10% *Coelastrella*; (2) 50% *Chlorella*/50% *Coelastrella*; and (3) 10% *Chlorella*/90% *Coelastrella*. Two experiments were run in parallel at two different temperatures: 27 °C and 37 °C. The temperatures were chosen in consideration of the hypothesis that the invasion by *Coelastrella* was due to a competitive advantage related to growth temperature optima. Thus, we chose the average temperature during the take-over (27 °C) and the optimum temperature for the *C. sorokiniana* 1230 strain (37 °C), from which *C. sorokiniana* 1228 was isolated (M. Huesemann, personal communication). For each experiment, cultures were inoculated into in 20 mL borosilicate glass tubes with 5 mL of BG11 media. Each treatment and control were set up with 6 replicates. Tubes were placed into the outer edge of tissue culture roller

drums (New Brunswick Scientific Co., Edison, NJ, USA) and drums were maintained at 80 RPM within growth chambers (Percival Scientific, IA, USA) that supplied light continuously at ~50 μmol $m^{-2} s^{-1}$ and CO_2 continuously at 1%.

On each sampling day, 200 μL was removed from each tube and analyzed via the FlowCam for relative abundance of *Chlorella* and *Coelastrella* as described in "Section 2.2 Outdoor Cultivation". In addition, cells were monitored microscopically using an Optima light microscope.

2.3.2. Exudate bioassay

In this assay, we cultivated each strain in media with added exudates that were extracted from previously-growing monocultures and co-cultures. To prepare exudates, each strain was first cultivated in monoculture for five days under the culture conditions described in "Section 2.1. Microalgae strain sources and characterization" except that temperature was maintained at 27 °C (818 Incubator, Precision Mechanical, Everett, WA, USA). These stock cultures were used to inoculate 550 mL tissue culture flasks (Polystrene, Grenier, NC, USA). Flasks of BG-11 media were inoculated to a final volume of 50 mL and final cell abundance of 6.64×10^5 cells mL^{-1} with either (1) monocultures of *Chlorella*; (2) monocultures of *Coelastrella*; or (3) a co-culture of *Chlorella* and *Coelastrella* at a 50/50 inoculum ratio by cell abundance. After six days of cultivation, cultures in the tissue culture flasks were harvested by centrifugation (5000 rpm, 5 min). A 50 mL aliquot of supernatant was collected from each culture, lyophilized, dissolved in 2 mL deionized water, and sterile filtered (0.2 μm PTFE syringe filter). This process resulted in concentrated exudates from the mono- and co-cultures.

We employed a 96-well microplate format [43–45] to culture *Chlorella* and *Coelastrella* monocultures in media conditioned with the exudates described above at varying percentages ranging from 6% to 100% (6%, 12.5%, 25%, 50%, 100%). For each culture and exudate concentration, 7 replicate wells were inoculated to a starting optical density at 750 nm (OD_{750}) of 0.1 with 100 μL of culture, 25 μL of diluted exudate, and 125 μL of BG11 medium. We also included a control that would not affect algal growth (25 μL of deionized water instead of exudates, 7 replicates) as well as a control that would affect algal growth (gentamicin at varying final concentrations, 3 replicates per plate). We used only the center of each 96-well plate (clear, flat bottom, Falcon, Corning, NY, USA) and filled the outside wells with media to prevent evaporation. Plates were wrapped with parafilm and maintained on an Orbit shaker (Lab-line Instruments, Mansfield, TX, USA) set to 75 RPM. The shaker was kept in a 27 °C-growth chamber (Percival Scientific, IA, USA) that supplied light continuously at 50 μmol $m^{-2} s^{-1}$ and CO_2 continuously at 1%. Optical density was measured daily for five days on a SpectraMax M2 (Molecular Devices, San Jose, CA, USA).

2.3.3. Scanning electron microscopy

To assess the nature of the physical association of co-occurring strains, we relied on scanning electron microscopy. Aliquots (~50 μL) of concentrated cultures were deposited on pre-cleaned 12 mm diameter glass coverslips, which were then immersed into 2 mL of 2.5% glutaraldehyde buffered with 0.1 M imidazole-HCl at pH 7.2 and sealed. The following day, the solution was aspirated off and replaced with the buffer solution for 20–30 min. Samples were dehydrated in a graded series of ethanol solutions (50%, 80%, absolute) and a final 1:1 (v/v) solution of absolute ethanol and hexamethyldisilazane (HMDS). This solution was replaced by HMDS and after several HMDS changes, samples were air-dried for 5 h. Coverslips were mounted on aluminum sample stubs using carbon adhesive tabs and colloidal silver paint (Electron Microscopy Sciences, Hatfield, PA) and examined in the high vacuum-secondary electron imaging mode of a model S-3400N Scanning Electron Microscope (Hitachi High Technologies, Dallas, TX). Digital images were collected for characterization of the cellular topography.

2.4. Calculations and statistical analyses

For the field cultivation trial and laboratory experiments, we used cell density and the FlowCam's Area Based Diameter algorithm to calculate biovolume of the two strains following the calculations for spherical cells [46]. Total biovolume was calculated as biovolume per cell x total cell number. Relative abundance of strains was calculated as percentages based on cell abundance (i.e., cell of an individual strain/total cell number in a co-culture) and biovolume (i.e., biovolume of an individual strain/total biovolume in a co-culture). For the exudate bioassay, we compared differences in absorbance on single sampling days or throughout the experiment (when there was no significant time x treatment interaction) using 1-way ANOVA with post-hoc Tukey tests in JMP v. 14. All data were log- transformed to meet the assumptions of normality and equal variance.

3. Results

3.1. Outdoor cultivation

After inoculation of *Chlorella sorokiniana* into the PBR, the culture displayed an apparent three to four-day lag followed by a four-day period of linear growth, as evidenced by the change in optical density through time (Fig. 1A). However, during this period, we observed the invasion and rapid culture take-over by an algal competitor later identified as *Coelastrella* sp. (Fig. 1B). One day after inoculation, the

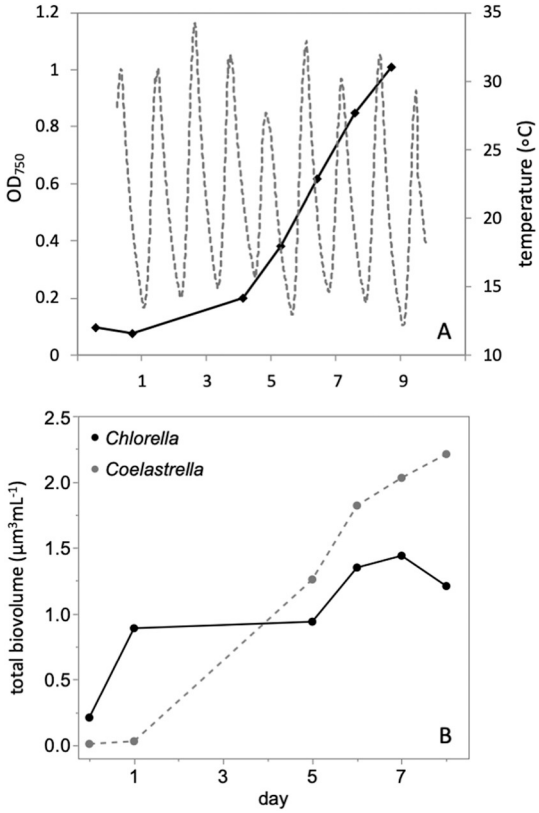

Fig. 1. Initial cultivation of *Chlorella sorokiniana* (UTEX1228) within an outdoor photobioreactor located at the Fabian Garcia Science Center in Las Cruces, NM. The top panel (A) shows the biomass (as optical density at 750 nm) of the inoculated culture as well as temperature within the photoreactor, and the bottom panel (B) shows the total biovolumes of *Chlorella sorokiniana* and the invading *Coelastrella* strain. In B, the initial data point showing no abundance of *Coelastrella* is assumed, given *Chlorella* was inoculated as a monoculture into an empty photobioreactor; all other points represent biovolumes measured from single samples collected on individual sampling days.

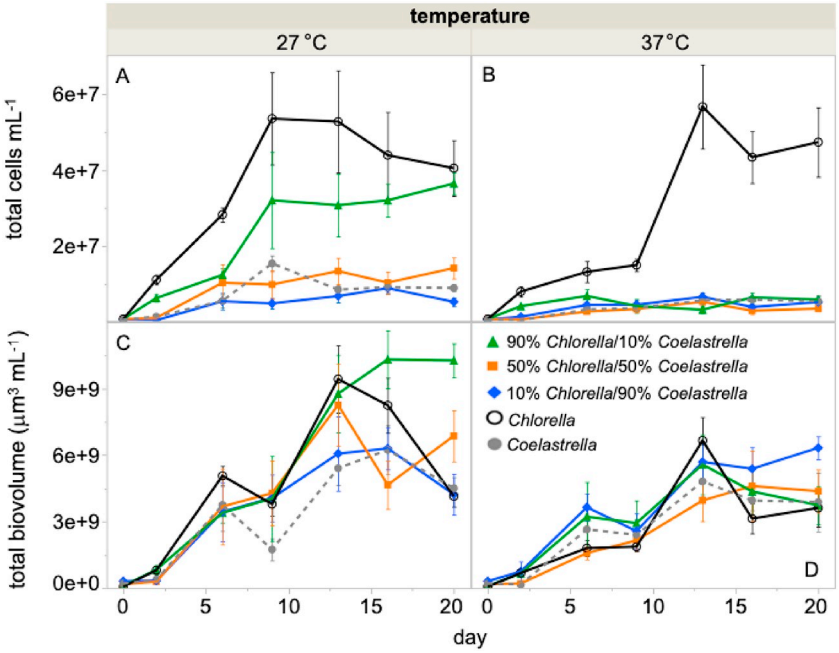

Fig. 2. Total cell abundance (top) and biovolume (bottom) in each of the co-culture treatments (initial inoculums of 90% *Chlorella*/10% *Coelastrella*; 50% *Chlorella*/50% *Coelastrella*; 10% *Chlorella*/90% *Coelastrella* by cell abundance) and monoculture controls during invasibility experiments conducted at 27 °C (left) and 37 °C (right). Points represent means (± standard errors) of six replicates.

invading *Coelastrella* was detected in the photobioreactor at relatively low biovolume. By the fifth day, *Coelastrella* dominated the culture in terms of biovolume (Fig. 1B). By day 8, a decrease in *Chlorella* biovolume was noted, indicating a potential crash. At the time of inoculation and scaling, temperature within the hoop-house ranged from 12 to 32 °C, with the daily average at ~21.5 °C (Fig. 1A).

3.2. Invasibility experiments

In the invasibility experiments, *Chlorella* monocultures displayed the greatest growth rates, with a characteristic log growth phase between days 0 and 10 (Fig. 2A–B). At 27 °C, cell abundance in cultures inoculated with 90% *Chlorella* also increased considerably during the first 10 days (Fig. 2A). Increases in cell number for all of the other treatments were either modest or moderate (Fig. 2A–B). Changes in biovolume generally followed changes in cell abundance, although slight differences between the patterns in biovolume and cell abundance were noted due to shifts in relative abundance of *Chlorella* and *Coelastrella* (Figs. 2, 3).

During both experiments, *Chlorella* and *Coelastrella* coexisted for twenty days (Fig. 3). *Coelastrella* tended to outcompete *Chlorella*, although there was an interactive effect of temperature and initial conditions in dictating the competitive dominant during the trials. At both temperatures, when *Coelastrella* was inoculated at high cell abundances (i.e., 10% *Chlorella*/90% *Coelastrella*), it remained the dominant strain in co-culture (Fig. 3E–F). Similarly, when it was inoculated at the same starting cell abundance as *Chlorella* (50% *Chlorella*/50% *Coelastrella*), it took hold and became the dominant strain after two or five days (Fig. 3C–D). At 37 °C, even when inoculated at low cell densities, *Coelastrella* outcompeted *Chlorella* by day 10 and then coexisted at relatively comparable cell numbers to *Chlorella* through the end of the experiment (Fig. 3B). An exception to this competitive dominance by *Coelastrella* was the 90% *Chlorella*/10% *Coelastrella* treatment at 27 °C, in which *Chlorella* remained dominant by cell number (Fig. 3A).

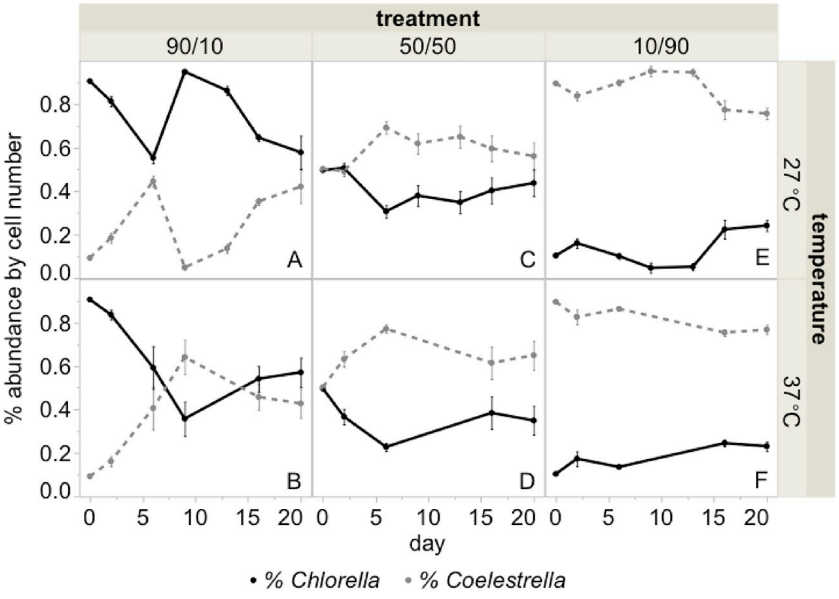

Fig. 3. Relative abundance of *Chlorella* and *Coelastrella* based on cell number in each of the co-culture treatments: 90% *Chlorella*/10% *Coelastrella* (left, A–B); 50% *Chlorella*/50% *Coelastrella* (middle, C–D); 10% *Chlorella*/90% *Coelastrella* (right, E–F) during invasibility experiments conducted at 27 °C (top) and 37 °C (bottom). Points represent means (± standard errors) of six replicates.

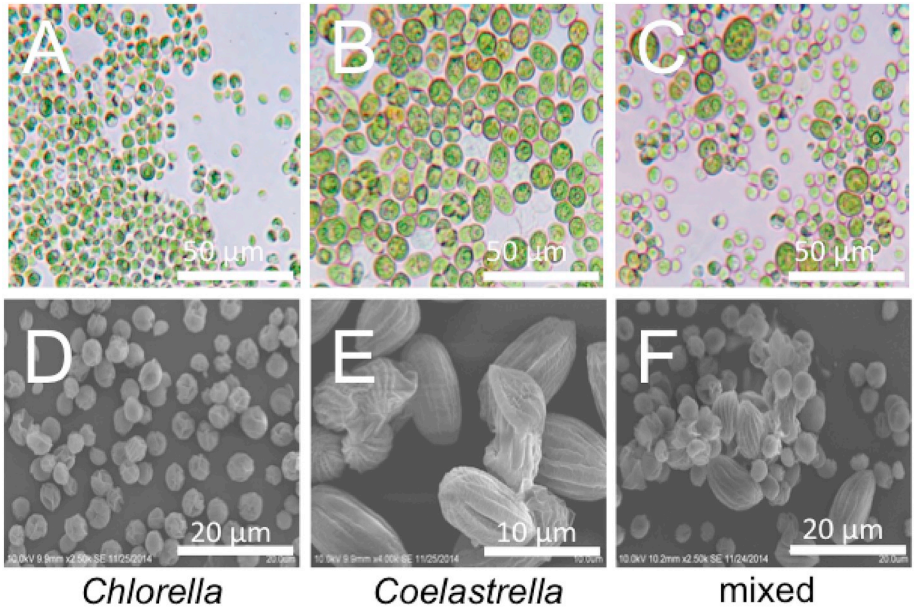

Fig. 4. Light (top) and Scanning Electron (bottom) micrographs of *Chlorella* monocultures (A, D), *Coelastrella* monocultures (B, E) and mixed *Chlorella* and *Coelastrella* cultures (C, F). Note that the *Coelastrella* SEM image has a different scale bar to better illustrate this organism's morphology.

However, this pattern is eliminated if biovolume is used as a metric of biomass, as *Coelastrella* is a much larger cell (Fig. S1, Fig. 4).

3.3. Exudate bioassay

Both *Chlorella* and *Coelastrella* exhibited log growth during the bioassay (Fig. 5). Notably, exudates from the *Chlorella* and *Coelastrella* co-cultures inhibited growth of *Chlorella* at the greatest extract concentrations of 50% and 100% (Fig. 5A). Indeed, on the final day of the assay, post-hoc Tukey tests revealed significant differences in OD_{750} between *Chlorella* cultures exposed to co-culture exudates 50% and 100% and those exposed to lower concentrations of co-culture exudate or no exudate (1-way ANOVA on day 4: $F(5) = 63.5$, $p < 0.001$; post hoc Tukey: $\alpha = 0.5$, $Q = 3.00$). In contrast, in the *Coelastrella* cultures exposed to co-culture exudate, the exudate did not negatively affect the growth of *Coelastrella* (Fig. 5B); there were no significant differences in OD_{750} between cultures exposed to different concentrations of these exudates during the assay (1-way ANOVA, $F(5) = 0.81$, $p > 0.05$). Similarly, the exudates from each of the monocultures did not generally illicit differences in growth in either *Chlorella* or *Coelastrella* cultures (Fig. 5C–F), although there was some evidence of inhibition by the *Chlorella* exudates, especially at the greatest exudate concentration (Fig. 5C–D). This inhibition was statistically significant for *Chlorella* cultures grown with *Chlorella* exudates (1-way ANOVA on day 4, $F(5) = 6.7$, $p < 0.001$), but not *Coelastrella* cultures exposed to *Chlorella* exudates (1-way ANOVA, $F(5) = 2.2$, $p = 0.05$). Following this trial, the entire assay was replicated with similar results (data not shown). In all cultures, the positive controls (i.e., cultures exposed to gentamicin) exhibited no or little growth, consistent with dosage (data not shown).

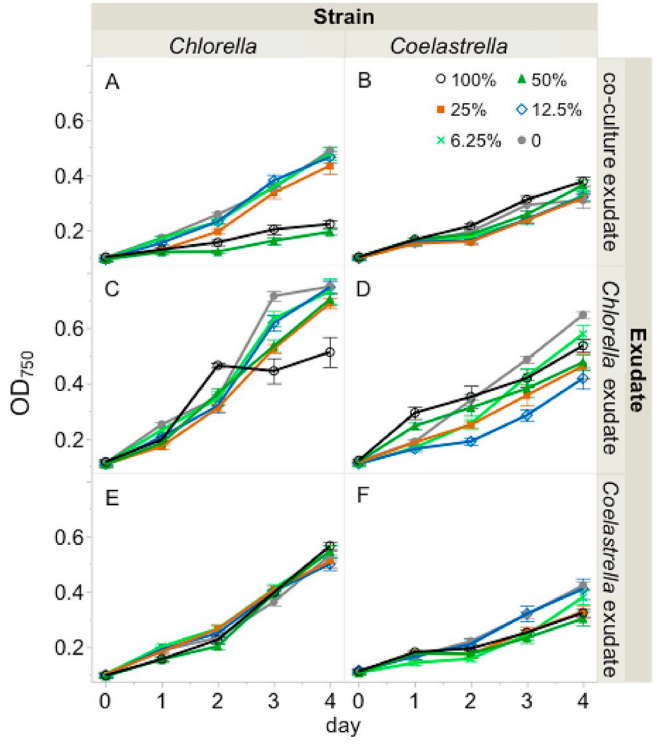

Fig. 5. Biomass as optical density at 750 nm of *Chlorella* (left) and *Coelastrella* (right) cultures grown in media containing various concentrations of exudates from previously scaled co-cultures (top, A–B), exudate from *Chlorella* monocultures (middle, C–D), and exudate from *Coelastrella* monocultures (bottom, E–F). Points represent means (± standard errors) of seven replicates.

4. Discussion

Understanding species interactions, especially those between established production strains of microalgae and invading pests, is critical to developing strategies and tools that will allow the microalgal cultivation industry to thrive. In this work, we attempted to scale a *Chlorella sorokiniana* strain in an outdoor photobioreactor, but scaling was halted by the invasion of an algal competitor. Pests such as these often invade algal cultures in the field, but pond uptime (i.e., the time before a production strain crashes) is typically much greater than a few days [9]. In this work, the invading algae outcompeted what appeared to be a monoculture of *Chlorella* quite rapidly – within just a few days. The invading culture, subsequently identified as a strain of *Coelastrella*, could have originated outdoors, as the taxon is ubiquitous globally [47]. Alternatively, it could have originated from the *Chlorella* culture itself; indeed, *Coelastrella* has been documented to invade other laboratory cultures of production strains [48]. However, contaminants

were not detected by either microscopy, flow cytometry, or sequencing prior to inoculation of the photobioreactor. Regardless of origin, after the *Coelastrella* contaminant was detected in the field, *Chlorella* growth virtually halted (Fig. 1B), despite its notable growth rates and tolerances to a variety of environmental conditions [49–51]. The termination of *Chlorella* growth in the field suggested that the *Coelastrella* strain was a competitive dominant.

In the laboratory, invasibility experiments confirmed that *Coelastrella* had a competitive advantage over *Chlorella* (Figs. 1 and 2), despite co-existence of the two strains for a period of at least 20 days. Possible mechanisms for coexistence in these experiments are numerous and could be resource-related (e.g. fluctuating resources through time, partitioning), strain-specific (e.g., fitness differences), or more complex. The competitive dominance of *Coelastrella* was especially apparent at higher temperatures, consistent with thermotolerance documented in other *Coelastrella* strains [52–54].

The exudate bioassay demonstrated a potential role for allelopathy in the competitive dominance by *Coelastrella* when co-cultured with *Chlorella*. Specifically, the assay demonstrated that exudates from co-cultures inhibited the growth of *Chlorella* monocultures (Fig. 4), a finding that could explain the rapid take-over of *Coelastrella* in the field. We hypothesize that our *Coelastrella* isolate is able to produce allelopathic chemical(s) that suppress the growth of *Chlorella sorokiniana*. Allelopathic interactions among phytoplankton strains are well documented. Early work demonstrated allelopathy in cultures of *Chlamydomonas reinhardtii* [55], *Chlorella vulgaris* [56], and *Anabaena cylindrica* [57,58]. Since then, many examples of allelopathic interactions among phytoplankton have emerged [e.g., 15,59,60], and recent work has expanded to consider whole food web interactions [61]. Notably, Ma et al. demonstrated the induction of temperature-dependent allelopathy in *Microcystis* when grown in the presence of *Chlorella* [27]. In this interaction, the growth of *Chlorella*, co-cultured with toxic or non-toxic *Microcystis* strains, was promoted at 20 °C but inhibited at temperatures ≥25 °C. Our invasibility experiments show a similar temperature advantage by *Coelastrella*, although we can not necessarily attribute this to an allelopathic effect, as we did not conduct exudate bioassays at multiple temperatures.

Of note in our exudate bioassay is the finding that exudates from *Coelastrella* monocultures did not inhibit *Chlorella* growth, whereas exudates from co-cultures did (Fig. 5). This finding suggests that any chemical(s) released by *Coelastrella* are not constitutively produced; instead, they are produced only when a microalgal competitor is present – highlighting the physiological or ecological cost of this response [62]. This suggestion is supported by the different molecular profiles of media from co-cultures compared to media from monocultures (data not shown). Induced responses have been documented in a variety of freshwater and marine algae, although a majority of the studies on induced responses has focused on defenses to grazers [63,64]. Still, there is a growing body of work that documents inducible changes in allelopathy in response to competitors [65,66]. For example, in a series of experiments focused on the dinoflagellate *Karenia brevis*, generally thought to constitutively produce toxins, Prince et al. [67] found that the presence of certain competitors altered the allelopathy of *K. brevis*. Specifically, the diatoms *Skeletonema costatum* and *Asterionellopsis glacialis* reduced the growth-inhibiting effects of bloom exudates by *K. brevis*. Likewise, the metabolism of competitors can change in response to the allelopathic organism; for example, using co-cultures of *K. brevis* and two diatoms, Poulson-Ellestad et al. [68] found no changes in the metabolic profile of *Asterionellopsis glacialis* – a robust competitor of *K. brevis*– but did find changes in the profile of *Thalassiosira pseudonana*, a poorer competitor. Using a model system, Dunker et al. [69] found that direct cell to cell contact with a green algal competitor was necessary for allelopathic growth of *Microcystis aeruginosa*. Broader (e.g., mechanical) defenses have also been shown to be inducible in response to the presence of a competitor. For example, Dong et al. [70] found that *Chlorella vulgaris* formed colonies with direct contact with the macrophyte and photosynthetic competitor *Ceratophyllum demersum*, and that high biomass of macrophytes caused the formation of more and larger colonies. More complex interactions have also been described. For example, Zhu et al. [71] showed that the presence of an algal competitor inhibited colony formation of *Scenedesmus obliquus* induced in response to grazers.

Our work is suggestive of the inducible production of chemicals in a microalgal strain in response to direct contact with a competing algal strain. Follow up studies, starting with isolation and characterization of allelopathic chemical(s), are needed to further develop this system between the two strains studied here. In addition, there are a number of experimental details that require attention. First, we were unable to rule out the possibility that the putative allelopathic chemical produced in the co-culture was exuded by *C. sorokiniana*. It could be that with the direct competition, *C. sorokiniana* produces chemical(s) either as an attempt to control *Coelastrella* or due to misreading of another chemical cue by *Coelastrella*. This strain does exhibit "clumping" of *C. sorokiniana* in the presence of *Coelastrella* (N. Nirmalakhandan, personal communication), illustrating responses to this organism. Second, studies examining allelopathy in different phases of growth and under different environmental conditions are warranted. In our exudate bioassays, there was some variability in the growth rates of our *Chlorella* controls due to our experimental design and plate locations within the incubator. Moreover, in both lab trials, in our assays, *Coelastrella* exhibited low growth rates in monoculture, when we suspect that allelochemicals were not produced. If energy was not going into chemical production, we would have expected higher growth rates. Finally, the suspected self-inhibition of *Chlorella* at the highest *Chlorella* exudate concentration is worth further investigation. Autoinhibition has previously been documented (Pratt and Fong 1940), but mechanistic understanding of this effect is still limited.

This work capitalized on a field crash of a microalgal production strain to understand species interactions. We demonstrated that the invading algae was a competitive dominate, likely through inducible allelopathic interactions. Yet, despite demonstrated allelopathy in phytoplankton communities, inducible allelopathic interactions are poorly understood. A closer look at these interactions in both natural systems as well as microalgal production facilities, with reliance on metabolomic and metagenomic tools, would contribute to our understanding of allelopathy in aquatic systems.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101535.

Acknowledgments

This study was supported in part by the National Science Foundation award #IIA-1301346; Department of Energy under contracts #DE-EE0006269; #DE-EE0006316. We acknowledge the assistance of Nicholas Csakan for the indoor/outdoor cultivation of *Chlorella sorokiniana* 1228 and Gary J. Chavez with cell enumeration using the FlowCAM.

Authors contributions

PJL, FOH, NN and WJB initiated and designed the project. MS and RN conducted the experiments. AAC conducted data analysis and wrote the manuscript. All authors contributed to ideas and revisions to the manuscript. WJB finalized the manuscript. All authors agree to authorship and approve the final manuscript for submission.

Conflict of interest statement

We declare that there are no conflicts of interest related to this work.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

References

[1] K. Kumar, S.K. Mishra, A. Shrivastav, M.S. Park, J.-W. Yang, Recent trends in the mass cultivation of algae in raceway ponds, Renew. Sust. Energ. Rev. 51 (2015) 875–885.

[2] D. Aitken, B. Antizar-Ladislao, Achieving a green solution: limitations and focus points for sustainable algal fuels, Energies 5 (2012) 1613–1647.

[3] J.W. Richardson, M.D. Johnson, J.L. Outlaw, Economic comparison of open pond raceways to photo bio-reactors for profitable production of algae for transportation fuels in the Southwest, Algal Res. 1 (2012) 93–100.

[4] L.T. Carney, T.W. Lane, Parasites in algae mass culture, Front. Microbiol. 5 (2014) 278.

[5] R.C. McBride, V.H. Smith, L.T. Carney, T.W. Lane, Crop protection in open ponds, in: S.P. Slocombe, J.R. Benemann (Eds.), Microalgal Production for Biomass and High-Value Products, CRC Press, Boca Raton, FL, 2016.

[6] V.H. Smith, R.C. McBride, Key ecological challenges in sustainable algal biofuels production, J. Plankton Res. 37 (2015) 671–682.

[7] P.J. Lammers, M. Huesemann, W. Boeing, D.B. Anderson, R.G. Arnold, X. Bai, M. Bhole, Y. Brhanavan, L. Brown, J. Brown, J.K. Brown, S. Chisholm, C. Meghan Downes, S. Fulbright, Y. Ge, J.E. Holladay, B. Ketheesan, A. Khopkar, A. Koushik, P. Laur, B.L. Marrone, J.B. Mott, N. Nirmalakhandan, K.L. Ogden, R.L. Parsons, J. Polle, R.D. Ryan, T. Samocha, R.T. Sayre, M. Seger, T. Selvaratnam, R. Sui, A. Thomasson, A. Unc, W. Van Voorhies, P. Waller, Y. Yao, J.A. Olivares, Review of the cultivation program within the National Alliance for Advanced Biofuels and Bioproducts, Algal Res. 22 (2017) 166–186.

[8] M. Hannon, J. Gimpel, M. Tran, B. Rasala, S. Mayfield, Biofuels from algae: challenges and potential, Biofuels 1 (2010) 763–784.

[9] R.L. White, R.A. Ryan, Long-term cultivation of algae in open-raceway ponds: lessons from the field, Ind. Biotechnol. 11 (2015) 213–220.

[10] Y. Chisti, Large-scale production of algal biomass: raceway ponds, in: F. Bux, Y. Chisti (Eds.), Algae Biotechnology: Products and Processes, Springer International Publishing, Cham, 2016, pp. 21–40.

[11] P.A. Lee, K.J.L. Martinez, P.M. Letcher, A.A. Corcoran, R.A. Ryan, A novel predatory bacterium infecting the eukaryotic alga *Nannochloropsis*, Algal Res. 32 (2018) 314–320.

[12] A.T. Ma, E.F. Daniels, N. Gulizia, B. Brahamsha, Isolation of diverse amoebal grazers of freshwater cyanobacteria for the development of model systems to study predator-prey interactions, Algal Res. 13 (2016) 85–93.

[13] E.L. Rice, Allelopathy - update, Bot. Rev. 45 (1979) 15–109.

[14] E.M. Gross, Allelopathy of aquatic autotrophs, Crit. Rev. Plant Sci. 22 (2003) 313–339.

[15] C. Legrand, K. Rengefors, G.O. Fistarol, E. Graneli, Allelopathy in phytoplankton - biochemical, ecological and evolutionary aspects, Phycologia 42 (2003) 406–419.

[16] R. Pratt, J. Fong, Studies on *Chlorella vulgaris* II further evidence that *Chlorella* cells form a growth-inhibiting substance, Am. J. Bot. 27 (1940) 431–436.

[17] A.T. Chan, R.J. Andersen, M.J. Leblanc, P.J. Harrison, Algal plating as a tool for investigating allelopathy among marine microalgae, Mar. Biol. 59 (1980) 7–13.

[18] S.L. Strom, Microbial ecology of ocean biogeochemistry: a community perspective, Science 320 (2008) 1043–1045.

[19] P.N. Leao, M. Vasconcelos, V.M. Vasconcelos, Allelopathy in freshwater cyanobacteria, Crit. Rev. Microbiol. 35 (2009) 271–282.

[20] K. Tan, Z. Huang, R. Ji, Y. Qiu, Z. Wang, J. Liu, A review of allelopathy on microalgae, Microbiology 166 (2019), https://doi.org/10.1099/mic.0.000776.

[21] A.B. Felpeto, S. Roy, V.M. Vasconcelos, Allelopathy prevents competitive exclusion and promotes phytoplankton biodiversity, Oikos 127 (2018) 85–98.

[22] S. Roy, Importance of allelopathy as pseudo-mixotrophy for the dynamics and diversity of phytoplankton, Biodiversity in Ecosystems - Linking Structure and Function, INTECH (2015) 17–28.

[23] R.M.W. Muhl, D.L. Roelke, T. Zohary, M. Moustaka-Gouni, U. Sommer, G. Borics, U. Gaedke, F.G. Withrow, J. Bhattacharyya, Resisting annihilation: relationships between functional trait dissimilarity, assemblage competitive power and allelopathy, Ecol. Lett. (2018) 1390–1400.

[24] R.X. Poulin, S. Hogan, K.L. Poulson-Ellestad, E. Brown, F.M. Fernández, J. Kubanek, *Karenia brevis* allelopathy compromises the lipidome, membrane integrity, and photosynthesis of competitors, Sci. Rep. 8 (2018) 9572.

[25] K.L. Poulson, R.D. Sieg, J. Kubanek, Chemical ecology of the marine plankton, Nat. Prod. Rep. 26 (2009) 729–745.

[26] M.D.C. Bittencourt-Oliveira, M.A. Chia, H.S.B. de Oliveira, M.K.C. Araújo, R.J.R. Molica, C.T.S. Dias, Allelopathic interactions between microcystin-producing and non-microcystin-producing cyanobacteria and green microalgae: implications for microcystins production, J. Appl. Ecol. 27 (2015) 275–284.

[27] Z. Ma, T. Fang, R.W. Thring, Y. Li, H. Yu, Q. Zhou, M. Zhao, Toxic and non-toxic strains of *Microcystis aeruginosa* induce temperature dependent allelopathy toward growth and photosynthesis of *Chlorella vulgaris*, Harmful Algae 48 (2015) 21–29.

[28] J.L. Allen, L. Ten-Hage, J. Leflaive, Regulation of fatty acid production and release in benthic algae: could parallel allelopathy be explained with plant defence theories? Microb. Ecol. 75 (2018) 609–621.

[29] H. Song, M. Lavoie, X. Fan, H. Tan, G. Liu, P. Xu, Z. Fu, H.W. Paerl, H. Qian, Allelopathic interactions of linoleic acid and nitric oxide increase the competitive ability of *Microcystis aeruginosa*, ISME J. 11 (2017) 1865–1876.

[30] J. Zhou, M.L. Richlen, T.R. Sehein, D.M. Kulis, D.M. Anderson, Z.H. Cai, Microbial community structure and associations during a marine dinoflagellate bloom, Front. Microbiol. 9 (2018) 1201.

[31] J. Zhou, Y. Lyu, M.L. Richlen, D.M. Anderson, Z. Cai, Quorum sensing is a language of chemical signals and plays an ecological role in algal-bacterial interactions, Crit. Rev. Plant Sci. 35 (2016) 81–105.

[32] L.B.B. Mendes, A.B. Vermelho, Allelopathy as a potential strategy to improve microalgae cultivation, Biotechnol. Biofuels 6 (2013) 152.

[33] M. Demuez, C. Gonzalez-Fernandez, M. Ballesteros, Algicidal microorganisms and secreted algicides: new tools to induce microalgal cell disruption, Biotechnol. Adv. 33 (2015) 1615–1625.

[34] A.K. Minhas, P. Hodgson, C.J. Barrow, A. Adholeya, A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids, Front. Microbiol. 7 (2016).

[35] P. Fergola, M. Cerasuolo, A. Pollio, G. Pinto, M. DellaGreca, Allelopathy and competition between *Chorella vulgaris* and *Pseudokirchneriella subcapitata*: experiments and mathematical model, Ecol. Model. 208 (2007) 205–214.

[36] M. DellaGreca, A. Zarrelli, P. Fergola, M. Cerasuolo, A. Pollio, G. Pinto, Fatty acids released by *Chlorella vulgaris* and their role in interference with *Pseudokirchneriella* subcapitata: experiments and modelling, J. Chem. Ecol. 36 (2010) 339–349.

[37] R.W. Castenholz, Culturing methods for cyanobacteria, Methods in Enzymology, Academic Press, 1988, pp. 68–93.

[38] M.M. Allen, Simple conditions for growth of unicellular blue-green algae, J. Gen. Microbiol. 51 (1968) 199–202.

[39] R.J. Andersen, M. Kawachi, Traditional microalgae isolation techniques, in: R.J. Andersen (Ed.), Algal Culturing Techniques, Elsevier Academic Press, Amsterdam, 2005.

[40] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[41] T. Selvaratnam, A.K. Pegallapati, F. Montelya, G. Rodriguez, N. Nirmalakhandan, W. Van Voorhies, P.J. Lammers, Evaluation of a thermo-tolerant acidophilic alga, *Galdieria sulphuraria*, for nutrient removal from urban wastewaters, Bioresour. Technol. 156 (2014) 395–399.

[42] M.L. Bartley, W.J. Boeing, D. Daniel, B.N. Dungan, T. Schaub, Optimization of environmental parameters for *Nannochloropsis salina* growth and lipid content using the response surface method and invading organisms, J. Appl. Phycol. 28 (2016) 15–24.

[43] J. Van Wagenen, S.L. Holdt, D. De Francisci, B. Valverde-Perez, B.G. Plosz, I. Angelidaki, Microplate-based method for high-throughput screening of microalgae growth potential, Bioresour. Technol. 169 (2014) 566–572.

[44] A.K. Franz, M.A. Danielewicz, D.M. Wong, L.A. Anderson, J.R. Boothe, Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity, ACS Chem. Biol. 8 (2013) 1053–1062.

[45] A.A. Corcoran, M.A. Saunders, A.P. Hanley, P.A. Lee, S. Lopez, R. Ryan, C.B. Yohn, Iterative screening of an evolutionary engineered *Desmodesmus* generates robust field strains with pesticide tolerance, Algal Res. 31 (2018) 443–453.

[46] H. Hillebrand, C.-D. Durselen, D. Kirschtel, U. Pollingher, T. Zohary, Biovolume calculation for pelagic and benthic microalgae, J. Phycol. 35 (1999) 403–424.

[47] M.D. Guiry, G.M. Guiry, AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 01 August 2018., (2018).

[48] A. Dawidziuk, D. Popiel, M. Luboinska, M. Grzebyk, M. Wisniewski, G. Koczyk, Assessing contamination of microalgal astaxanthin producer *Haematococcus* cultures with high-resolution melting curve analysis, J. Appl. Genet. 58 (2017) 277–285.

[49] C. Sorokin, R.W. Krauss, Maximum growth rates of *Chlorella* in steady-state and in synchronize cultures, Proc. Natl. Acad. Sci. U. S. A. 45 (1959) 1740–1744.

[50] F.W. Wagner, B.E. Welch, Oxygen-tolerant strain of *Chlorella sorokiniana*, Appl. Microbiol. 17 (1969) 139–144.

[51] J. Masojídek, G. Torzillo, Mass cultivation of freshwater microalgae, Reference Module in Earth Systems and Environmental Sciences, Elsevier, 2014.

[52] P.H. Chiu, K. Soong, C.N.N. Chen, Cultivation of two thermotolerant microalgae under tropical conditions: influences of carbon sources and light duration on biomass and lutein productivity in four seasons, Bioresour. Technol. 212 (2016) 190–198.

[53] C.W. Hu, L.T. Chuang, P.C. Yu, C.N.N. Chen, Pigment production by a new thermotolerant microalga *Coelastrella* sp F50, Food Chem. 138 (2013) 2071–2078.

[54] G.S. Narayanan, G. Kumar, S. Seepana, R. Elankovan, S. Arumugan, M. Premalatha, Isolation, identification and outdoor cultivation of thermophilic freshwater microalgae *Coelastrella* sp. FI69 in bubble column reactor for the application of biofuel production, Biocatal. Agric. Biotechnol. 14 (2018) 357–365.

[55] V.W. Proctor, Studies of algal antibiosis using *Haematococcus* and *Chlamydomonas*, Limnol. Oceanogr. 2 (1957) 125–139.

[56] W. Rodhe, Environmental requirements of fresh-water plankton algae, Symb. Bot. Upsal. 10 (1948) 1–149.

[57] G.E. Fogg, D.F. Westlake, The importance of extracellular products of algae in freshwater, SIL Proceedings, 1922–2010, 12 1953, pp. 219–232.

[58] G.E. Fogg, D.F. Westlake, The importance of extracellular products of algae in freshwater, Mitt. Int. Ver. Theor. Angew. Limnol. 12 (1955) 219–232.

[59] A. Ianora, M.G. Bentley, G.S. Caldwell, R. Casotti, A.D. Cembella, J. Engstrom-Ost, C. Halsband, E. Sonnenschein, C. Legrand, C.A. Llewellyn, A. Paldaviciene, R. Pilkaityte, G. Pohnert, A. Razinkovas, G. Romano, U. Tillmann, D. Vaiciute, The relevance of marine chemical ecology to plankton and ecosystem function: an emerging field, Mar. Drugs 9 (2011) 1625–1648.

[60] A.B. Felpeto, V.M. Vasconcelos, Allelopathic interactions in phytoplankton

population ecology, J. Allelochem. Inter. 2 (2016) 25–34.
[61] G. Franzè, J.J. Pierson, D.K. Stoecker, P.J. Lavrentyev, Diatom-produced allelochemicals trigger trophic cascades in the planktonic food web, Limnol. Oceanogr. 63 (2018) 1093–1108.
[62] R. Tollrian, C.D. Harvell, The Ecology and Evolution of Inducible Defenses, Princeton University Press, Princeton, 1999.
[63] E. Van Donk, A. Ianora, M. Vos, Induced defences in marine and freshwater phytoplankton: a review, Hydrobiol 668 (2011) 3–19.
[64] M. Pancic, T. Kiorboe, Phytoplankton defence mechanisms: traits and trade-offs, Biol. Rev. 93 (2018) 1269–1303.
[65] H. Ben Gharbia, O. Kéfi-Daly Yahia, P. Cecchi, E. Masseret, Z. Amzil, F. Herve, G. Rovillon, H. Nouri, C. M'Rabet, D. Couet, H. Zmerli Triki, M. Laabir, New insights on the species-specific allelopathic interactions between macrophytes and marine HAB dinoflagellates, PLoS One 12 (2017) e0187963.
[66] D.B. Rasher, M.E. Hay, Competition induces allelopathy but suppresses growth and anti-herbivore defence in a chemically rich seaweed, Proc. R. Soc. Lond. B Biol. Sci. 281 (2014) 20132615.
[67] E.K. Prince, T.L. Myers, J. Naar, J. Kubanek, Competing phytoplankton undermines allelopathy of a bloom-forming dinoflagellate, Proc. R. Soc. Lond. B Biol. Sci. 275 (2008) 2733.
[68] K.L. Poulson-Ellestad, C.M. Jones, J. Roy, M.R. Viant, F.M. Fernandez, J. Kubanek, B.L. Nunn, Metabolomics and proteomics reveal impacts of chemically mediated competition on marine plankton, Proc. Natl. Acad. Sci. U. S. A. 111 (2014), https://doi.org/10.1073/pnas.1402130111.
[69] S. Dunker, J. Althammer, G. Pohnert, C. Wilhelm, A fateful meeting of two phytoplankton species - chemical vs. cell-cell-interactions in co-cultures of the green algae *Oocystis marsonii* and the cyanobacterium *Microcystis aeruginosa*, Microb. Ecol. 74 (2017) 22–32.
[70] J. Dong, Y. Gao, M. Chang, H. Ma, K. Han, X. Tao, Y. Li, Colony formation by the green alga *Chlorella vulgaris* in response to the competitor *Ceratophyllum demersum*, Hydrobiologia 805 (1) (2018) 177–187 (2018).
[71] X. Zhu, J. Wang, Y. Lu, Q. Chen, Z. Yang, Grazer-induced morphological defense in *Scenedesmus obliquus* is affected by competition against *Microcystis aeruginosa*, Sci. Rep. 5 (2015) 12743.