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Bacterial communities protect the alga *Microchloropsis salina* from grazing by the rotifer *Brachionus plicatilis*

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ABSTRACT

Open algal ponds are likely to succumb to unpredictable, devastating crashes by one or several deleterious species. Developing methodology to mitigate or prevent pond crashes will increase algal biomass production, drive down costs for algae farmers, and reduce the risk involved with algae cultivation, making it more favorable for investment by entrepreneurs and biotechnology companies. Here, we show that specific algal-bacterial co-cultures grown with the green alga *Microchloropsis salina* prevented grazing by the marine rotifer, *Brachionus plicatilis*. We obtained seven algal-bacterial co-cultures from crashed rotifer cultures, maintained them in co-culture with *Microchloropsis salina*, and used a microalgal survival assay to determine that algae present in each co-culture were protected from rotifer grazing and culture crash. After months of routinely diluting and maintaining these seven algal-bacterial co-cultures, we repeated the assay and found the opposite result: none of the seven bacterial communities protected the microalgae from rotifer grazing. We performed 16S rRNA gene amplicon sequencing on the protective and nonprotective co-culture samples and identified substantial differences in the makeup of the bacterial communities. Protective bacterial communities consisted primarily of Alphaproteobacteria (Rhodobacteraceae) and Gammaproteobacteria (*Marinobacter*, *Pseudomonas*, *Methylophaga*) while nonprotective bacterial communities were less diverse and missing many putatively crucial members. We compared the seven protective communities with the seven nonprotective communities and we correlated specific bacterial amplicon sequence variants with algal protection. With these data, our future work will aim to define and develop an engineered-microbiome that can stabilize industrial *Microchloropsis salina* cultures by protecting against grazer-induced pond crashes.

1. Introduction

One major problem facing algal production systems is unpredictable and complete loss of an algal crop. A 'pond crash' can be caused by a wide range of factors, including weather, water chemistry, contamination by other algae, infection, and grazing [1,2]. Closed algal photobioreactor systems are less prone to contamination (by viruses, fungi, protozoans, detrimental microbes, etc.) but are difficult to decontaminate when they do become infected and they require a high capital cost [1,3]. Open algal ponds require substantially lower capital costs but are more prone to contamination and are more likely to succumb to crashes due to one or several deleterious species [4]. Annually, pond crashes account for up to 30% loss of algal yield [5], which substantially drives up the cost per unit of biofuel production.

Developing methodologies to address pond crashes is critical for continued efforts in algal cultivation. Recently, high-throughput amplicon sequencing (Illumina) has been used to identify eukaryotic taxa associated with algal pond crashes that may serve as early bio-indicators of an impending crash [2]. Similarly, others have investigated algal pond pests to further characterize detrimental biological invaders [5]. Additionally, the inefficacy of most current practices invites the development of innovative intervention strategies towards algal pond

Abbreviations: ASV, amplicon sequence variant; bp, base pair; rRNA, ribosomal ribonucleic acid; p, protective; n, nonprotective; RFU, relative fluorescence unit; R, rotifer

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pest control. Standard industrial strategies are largely focused on physical methods (e.g., filtration and sonication) [4] and the addition of various chemicals agents (e.g., quinine, formaldehyde, ammonia, and hydrogen peroxide) [6] to monocultures of algae to prevent or treat pond crashes. These chemical additives are often expensive, require routine re-applications due to photo- or biodegradation (e.g., Rotenone) [7], and have unacceptable off-target effects including harm to algal crops, change of pond chemical composition, and ecotoxicity to wildlife. New methods and updated biotechnologies are necessary for continuous, dependable, and persistent crop protection to reduce algal pond loss.

Recent efforts in agriculture, aquaculture, and human medicine have explored the potential effectiveness of microbiome restoration or manipulation towards combating disease or loss of function [8–10]. There is a wealth of understanding from the aquatic microbial ecology field relating to the beneficial interactions between microalgae and bacteria, including for example, increased growth via remineralization of nutrients [11], exchange of essential vitamins [12], and increased lipid and bioproduct synthesis [13]. Yet in industrial algal cultivation, the algal microbiome – the bacterial community associated with algal cultures – has been largely undervalued and accordingly understudied. However, numerous studies support the idea that certain 'probiotic' bacterial strains could provide benefits in algal cultivation, specifically to protect against grazing and stabilize algal systems. The bacterially-produced alkaloid violacein inhibits growth and survival of several freshwater ciliates, flagellates, and rotifers [14]. Similarly, prodigiosin, a secondary metabolite produced by *Pseudoalteromonas rubra*, acts as a chemical defense system for the microbe. Indeed, *P. rubra* along with *P. piscicida*, *P. luteoviolacea*, and members of the Photobacteriaceae and Vibrionaceae families have been found to have marked toxicity to model eukaryotes [15].

Here, we present data that supports the use of bacterial communities in algal growth production ponds, as a novel approach to mitigate pond crashes. We selected the marine rotifer *Brachionus plicatilis*, which is capable of consuming 200 microalgal cells per minute and doubling in population within 1–2 days [16], as our model. Our intent with this work was to determine if bacterial communities can reduce grazing of *Microchloropsis salina* by the marine rotifer, *Brachionus plicatilis*. Such pest mitigation approaches utilizing protective algal-bacterial co-cultures could potentially be implemented without the additional expense of standard interdiction techniques. We aim to understand the implications of the algal microbiome to support industrial algal production systems.

2. Materials and methods

2.1. Axenic algae and rotifer cultures

Axenic stock culture of *Microchloropsis salina* CCMP 1776 was purchased from the National Center for Marine Algae and Microbiota (NCMA at Bigelow Laboratory, ME, USA); axenicity was defined by NCMA standards and *M. salina* cultures were not tested. Axenic *M. salina* and co-cultures of *M. salina* and bacteria were grown in modified ESAW medium [17] containing 1.65×10^{-3} M nitrate and 6.72×10^{-5} M phosphate and no silica at 20 °C, a light intensity of 100 μmol $m^{-2} s^{-1}$ and with a 16:8 h light:dark cycle. The cysts of marine, L-type rotifer *Brachionus plicatilis* were purchased in a batch of 20,000 cysts per vial (Florida Aqua Farms, FL, USA) and were sterilized by suspending in 0.5 mg L^{-1} sodium hypochlorite for 60 min at room temperature, pelleted, suspended in 0.25 mg L^{-1}sodium hypochlorite for 30 min, pelleted, resuspended in 20 mL ESAW in a sterile 100 mm petri dish, incubated for 48 h under lamps at 28 °C, and then grown axenically at room temperature (about 22 °C) in 1 L of sterile ESAW [18]. Rotifer cultures were fed approximately 3×10^8 cells axenic *M. salina* every other day and washed approximately every 14 d for culture maintenance. Rotifer density was determined by light microscopy counts.

2.2. Algal-bacterial co-culture sources, enrichment, and culturing

Xenic *B. plicatilis* were hatched by the described method, above, without the hypochlorite treatment. Two 1-L cultures of xenic rotifers were fed with axenic *M. salina* and grown in semi-continuous culture in ESAW for approximately 30 days (at about 100 rotifers mL^{-1}) when they crashed, as evidenced by failure to consume algae and the absence of motile rotifers. The bacterial fraction was harvested from each of these cultures by sequential passage through 0.8-μm cellulose nitrate Nalgene filters (ThermoFisher Scientific, MA USA) and collection on 0.2-μm polyethersulfone membrane filters (VWR, PA, USA). Bacterial fractions (1–4) harvested from 1-L of rotifer culture were resuspended in 150 mL ESAW from which 20 mL was concentrated to 2.5 mL on a 0.2-μm filter and then divided between duplicate flasks of axenic cultures of *M. salina* (25 mL at 5×10^5 algae cells mL^{-1}). The co-cultures were maintained in semi-continuous culture under standard growth condition. Additional algal-bacterial co-cultures (5–7) were established in a similar fashion from laboratory rotifer cultures that suffered catastrophic crash. Cultures were routinely diluted at 1:100 every 2 weeks. In this way, seven algal-bacterial co-cultures were collected and maintained.

Axenic *M. salina* was added to these challenged co-cultures to maintain an algal concentration of 10–15 M mL^{-1} with media replacement every 2 weeks for 1 month. After this challenge phase, the remaining live and dead rotifers were removed and the algal-bacterial co-cultures were maintained via weekly ESAW dilutions (1:10). Flask-based grazing assays were performed (as described below) on the algal-bacterial co-cultures after the 30-day rotifer challenge and again after about 75 days of growth without *B. plicatilis* present.

Freezer stocks of the algal-bacterial co-cultures were prepared by directly mixing with 50% DMSO-50% ESAW preservation solution and stored at −80 °C immediately before each microalgal growth assay. To re-start a culture from a frozen stock, the cryovial was allowed to warm to room temperature (about 22 °C) and then was added to a flask containing 25 mL of ESAW with 1–5 M cells mL^{-1} of axenic *M. salina*. Cultures were routinely diluted as stated previously and grown for four weeks at standard conditions at which point rotifers were added at 10–20 rotifers mL^{-1}. More axenic *M. salina* was added to these challenged co-cultures as necessary and cultures were maintained as previously described for six weeks before assayed.

2.3. Microalgal growth assay

To quantitatively assess the protective capacities of the algal-bacterial co-cultures, we monitored densities of algal culture inoculated with bacterial cultures 1–7 over the co-cultures' growth phase. Six replicate cultures (25 mL) of 1–7 and untreated *M. salina* were set up in 125-mL baffled Erlenmeyer flasks at dilutions of $2–6 \times 10^6$ algal cells mL^{-1} in ESAW. After 2 days of growth, axenic *B. plicatilis* were added to three of the six flasks for each culture at 10 rotifers mL^{-1} (250 rotifers per flask). Prior to addition, rotifers were concentrated using 30-μm filters and the concentrated stock was fixed with 15 μL of 1 M acetic acid counted twice using a 1-mL hemacytometer. Flasks were shaken at 65 rpm and grown under standard conditions (see Section 2.1). As a measure of algal density and growth, daily timepoint chlorophyll fluorescence readings (430 nm excitation, 685 nm emission) were taken in duplicate from the 48 individual flasks (200-μL subsamples) over 10–11 days for each experiment using a Tecan i-control infinite 200Pro version 1.11.1.0. This assay was conducted within 30 days of establishing the algal-bacterial co-cultures and repeated after 75 days of cultivation in the absence of rotifers.

Prior to the first flask-based microalgal growth assay, the seven algal-bacterial co-cultures were screened for protection of microalgae using a 24-well plate (ThermoFisher Scientific, MA USA). For this assay,

Table 1
Normalized average final RFU for microalgal growth assay with *B. plicatilis* comparing bacterial communities with *M. salina* (1–7) to untreated *M. salina* (con).

	Con	1	2	3	4	5	6	7
Final normalized algal density	0.9	17.6	65.6	70.0	11.3	16.8	74.1	17.1
Final motile rotifer count	50 +	7	1	4.5	10.5	4	1.5	12

similar experimental conditions (as in the flask assay) were maintained except that 10 rotifers mL^{-1} were added on the initial day of the experiment, only 1 mL volumes of algal-bacterial co-cultures were added per well for each co-culture 1–7 and the untreated *M. salina* control, and daily chlorophyll fluorescence was collected for 7 days. Similarly, another plate assay was performed after growing algal-bacterial stocks from frozen aliquots. For this experiment, the same procedure for the plate assay was followed except there were duplicate samples (n = 2) for 'no rotifer control' samples and n = 4 for algal-bacterial co-cultures in the presence of rotifers.

2.4. Calculations and statistical analysis

Duplicate fluorescence measurements for each sample were averaged for each timepoint and then normalized to the final algal density of the untreated *M. salina* control without rotifers (con) for each experiment, except for the plate assay. Since the 24-well plate assay did not have a 'no rotifer' untreated *M. salina* control, all samples were normalized to the highest fluorescence value collected, which was day 6 of co-culture 3 with rotifers (see Table 1). Specific algal growth rates (μ) were calculated as the slope over of the natural log of fluorescence data for each sample [19], averaged, and then normalized to the untreated *M. salina* control without rotifers (con) for each experiment. Since the intent of the experiment was to understand the effect of rotifers on algal growth, we only determined "μ" for the period from 24 h after rotifer addition through the end of each experiment (days 3–10 for 1p-7p and days 3–9 for 1n-7n). All timepoints and specific growth rates are shown as the average of three biological replicates with error bars equal to the standard deviation (n = 3 for flask-based assays). For the plate-based microalgal growth assay in Fig. 5, error bars are shown as standard deviations for n = 2 (no rotifer control samples) and n = 4 (samples with rotifers added).

2.5. DNA extraction

To prepare samples for 16S rRNA gene amplicon sequencing, algal-bacterial co-culture samples were thawed and extracted for genomic DNA using the Quick-DNA Fungal/Bacterial 96 Kit (Zymo Research, Irvine, CA, USA), following manufacturer's instructions with the addition of a bead-beating step (1750 rpm, 2× 30s). Extracted DNA was eluted with 2 × 25 μL Ultra-Pure water. DNA concentrations were quantified by High Sensitivity Quant-iT dsDNA Assay Kit (480 nm excitation, 530 nm emission; ThermoFisher Scientific, MA USA).

2.6. Library preparation and sequencing

The V3/V4 sequencing libraries were prepared via two-step PCR. First, the V3/V4 hypervariable regions of the prokaryotic 16S ribosomal DNA were amplified using primers 357F and 783R [20,21] designed to exclude chloroplasts. The first PCR amplification reaction contained 5 μL of 10X AccuPrime Pfx mix (ThermoFisher Scientific, MA USA), 0.3 μM final concentration of each primer, 0.4 μL of AccuPrime Pfx polymerase, and 25 ng of genomic DNA in a final reaction volume of 50 μL. The cycling conditions were an initial denaturation at 95 °C for 3 min; 24 cycles of 95 °C for 30 s, 50 °C for 45 s, and 68 °C for 1 min; and a final 7-min extension at 68 °C. PCR products were cleaned and concentrated to 25 μL using a Zymogen DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). A second round of PCR was performed to add Illumina adapter sequences to the 5′ of amplicons, per manufacturer's protocol (16S Metagenomic Sequencing Library preparation, Illumina). PCR products were cleaned and concentrated to 25 μL using a Zymogen DNA Clean & Concentrator kit, as above.

Illumina-barcoded amplicon libraries were quantified using a Quant-iT dsDNA Assay Kit, as above, after which 10 ng of 96 samples were pooled, cleaned and concentrated to 20 μL using the Zymogen Clean and Concentrate kit, as above. Prior to sequencing, the average library DNA size was determined to be 585 bp (2100 Bioanalyzer; Agilent, Santa Clara, CA, USA). The denatured library was diluted to 6 pM in HT1 buffer (Illumina) and mixed with PhiX (6 pM). Paired-end 300-bp sequencing was performed on an Illumina MiSeq with v3 chemistry (Illumina, San Diego, CA, USA) at Sandia National Laboratory (Livermore, CA).

2.7. Bioinformatic analysis of SSU rRNA gene amplicon sequencing

Ribosomal amplicon sequence variants (ASVs) were determined using DADA2 version 1.6.0 [22]. Briefly, quality-filtered reads were trimmed from both the 5′ to remove primers and 3′ to remove low-quality nucleotide calls. Read pairs were then denoised based on a DADA2 error model, merged, and de novo chimeras were removed. The final output consisted of the final ASV sequences of 16S rRNA genes and an ASV table of the number of reads per ASV per sample (functionally analogous to an 'OTU table'). Taxonomy of ASVs was assigned using Ribosomal Database Project (RDP) Classifier v 2.11 with Release 11.5 [23]. ASVs assigned either to the 'Chloroplast' taxonomic class or identified as algal mitochondrial sequences were removed, as well as ASVs not counted at least 5 times in at least one sample. Sequence analysis was done in R [24] primarily using Phyloseq 1.22.3 [25]. Correlations between ASVs and with protection, as measured by algal specific growth rates in the presence of rotifers, were determined using Spearman's rank-order correlation and p-values were adjusted for multiple testing with Benjamini-Hochberg correction.

3. Results and discussion

3.1. Algal-bacterial co-cultures protective against rotifer grazing

The algal-bacterial co-cultures (henceforth referred to as 1–7) were initially screened for protective qualities by assessing algal clearance by rotifers. When high algal densities were evident, samples were examined under low-magnification light microscopy and 1–7 contained several dead or impaired rotifers (see supplemental videos). Encouraged by this observation, 1–7 were screened using a 24-well plate microalgal growth assay in order to quantitatively assess protection of each algal-bacterial co-culture relative to an untreated *M. salina* control culture (Table 1). Co-cultures 1–7 exhibited very few living rotifers compared to the control, which had at least 50 rotifers present and swimming normally (Table 1). Thus, co-cultures 1–7 were categorized as "protective" co-cultures, henceforth denoted 1p-7p.

After this first screen, we developed a more robust microalgal growth assay, with larger sample sizes and in biological triplicate, to collect higher quality data to assess the protection of co-cultures 1p-7p in comparison to an untreated *M. salina* control culture in the absence and the presence of rotifers (Fig. 1). When rotifers were added on day 2, algal-bacterial co-cultures 1p-7p recovered after a 2–3-day lag and ultimately escaped grazing, while the untreated *M. salina* control failed to recover (Fig. 1A). After day 3, algal growth was roughly linear over the entire time course and algal growth rates were determined and compared (Fig. 1B). When challenged by rotifers, 1p-7p conferred higher algal growth rates relative to the untreated *M. salina* control. It is worth noting that although 2p was not significant at $p < 0.05$, it still had a

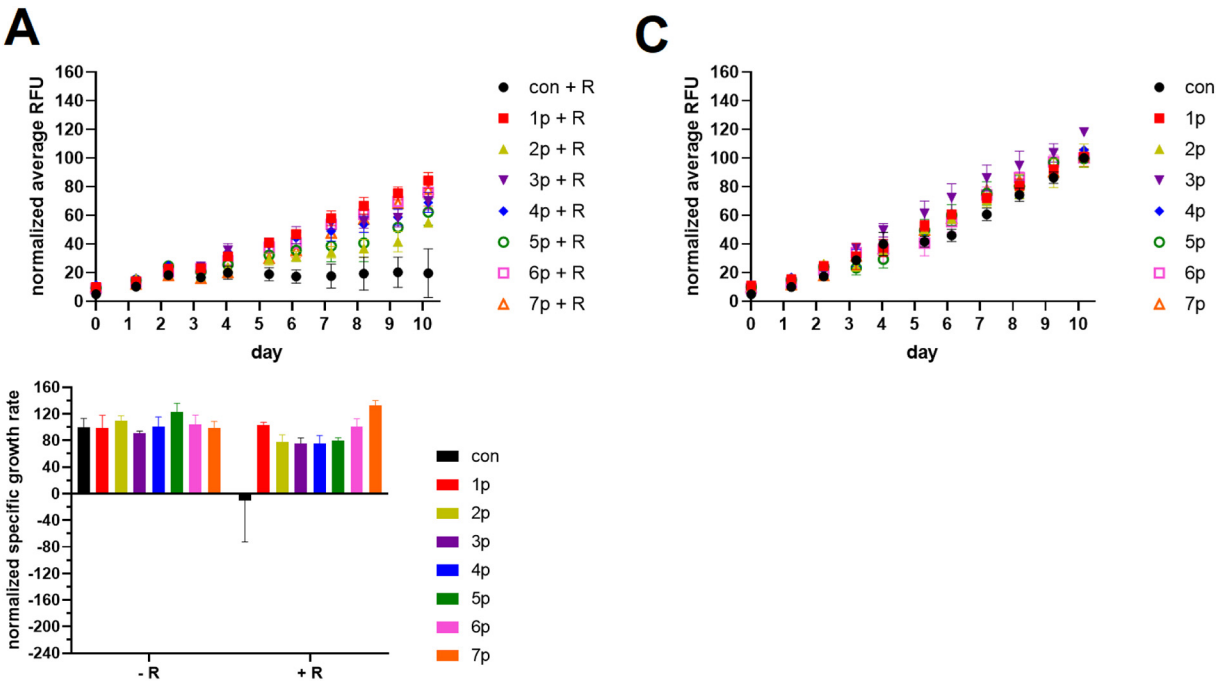

Fig. 1. Protective bacterial communities (1p-7p) protect *M. salina* from culture crash in presence of rotifers (+R) (A), show positive specific growth rates (μ), similar to control culture (con) and co-cultures (1p-7p) without rotifers (B), and do not inhibit *M. salina* growth in the absence of rotifers (C). All graphs show normalized replicate growth (n = 3) as measured by chlorophyll fluorescence in relative fluorescence units (RFU).

higher average growth rate and prevented its algal culture from crashing in the presence of rotifers (Fig. 1A).

The growth of the 1p-7p in the absence of rotifers was very similar to that of the untreated algal culture (Fig. 1C), thus supporting that the bacteria do not have detrimental effects on algal growth. Further, any notable growth increases in the absence of rotifers were poorly correlated with protection (growth in the presence of rotifers; Pearson's $r = 0.31$, $p = 0.21$). These results, in combination with our initial results in Table 1, indicated that the potential mechanism of "protection" was impairment of rotifer viability or reproduction and not limited to a probiotic effect on algal growth. This protective phenotype can be attributed to a microbial-based effect directly on the rotifers (perhaps chemical or pathogen in nature) or through induction of algal defenses.

3.2. Loss of algal protection after cultivation in the absence of rotifers

After approximately 75 days of routinely diluting and maintaining the algal-bacterial co-cultures 1–7 without rotifers present, the microalgal growth assay was repeated and we found that the bacteria in the cultures were no longer protective (Fig. 2). After rotifers were added on day 2, the algal growth again lagged for 2–3 days until the cultures ultimately crashed (Fig. 2A). Despite consistent experimental conditions, the algal-bacterial co-cultures were clearly no longer protective against rotifer grazing of *M. salina*. These nonprotective co-cultures of 1–7 are henceforth referred to as 1n-7n. Although 1n-7n descended from the original algal-bacterial co-cultures, 1–7, that resulted in dead or impaired rotifers (Table 1), in this second iteration of the flask-based microalgal growth assay, the untreated algal control and algal-bacterial co-cultures 1n-7n all exhibited negative algal growth and ultimately crashed (Fig. 2A, B).

Interestingly, in the absence of rotifers, 3n exhibited 28.1% higher algal productivity than the untreated control culture (Fig. 2B). This result that some bacteria can enhance microalgal growth is not novel, but the application of these bacteria for algal production systems has only recently been suggested and reviewed (for review see [26]). Our results support that growth improvements from the co-cultures alone cannot explain protection from rotifer grazing and that probiotic effects on algal growth are independent of protection.

3.3. Microbial community analysis

Using 1p-7p and 1n-7n co-cultures saved immediately prior to microalgal survival assays, we performed 16S amplicon sequencing in order to compare the microbial communities of 1p-7p and 1n-7n to examine broad bacterial community changes and identify members that changed in abundance. The 1p-7p bacterial communities were quite similar with an average Bray-Curtis dissimilarity of 0.26 (range of 0.11–0.41). They consisted almost entirely of Proteobacteria taxa, with 60–86% of the libraries containing unclassified Rhodobacteraceae and *Marinobacter* genera (Fig. 3). The most abundant genus identified within Rhodobacteraceae consisted primarily of three abundant ASVs that could not be definitively assigned due to high similarity (99–100%) to multiple reference sequences. These taxa belong to the Roseobacter clade within Rhodobacteraceae, which often make up a large portion of ocean bacterioplankton communities [27] and are known to commonly associate with marine microalgae [28,29]. Roseobacters have previously been observed in biofuel microalgal cultures [30], including those of *M. salina* [31]. *Marinobacter* (15–49% abundance) consisted of two abundant ASVs with similarity to *Marinobacter alkaliphilus* (99–100% identity) and was the second most abundant genus for all but one of the protective communities in 1p-7p. *Marinobacter*, ubiquitous throughout marine systems [32], is also commonly found in close proximity to chlorophytes [33] and other algae [34].

Pseudomonas had a slightly higher relative abundance (26%) in 4p, although it was not present (> 1%) in all protective communities. *Methylophaga* was present in 1p-7p but at consistently low relative abundance (2.7–4.4%). Other lower-abundance genera intermittently present in protective communities included the Alphaproteobacteria genera *Ruegeria*, *Paracoccus*, and *Phenylobacterium*, a Cytophaga genus *Marinoscillum*, and an Actinobacteria genus *Dietzia* (Fig. 3B). Most of these genera, or closely related groups, have previously been observed growing in association with microalgae [35,36], which is relevant considering the bacteria were maintained in co-culture with *M. salina*, with the bacteria's organic carbon supply (aside from the vitamins

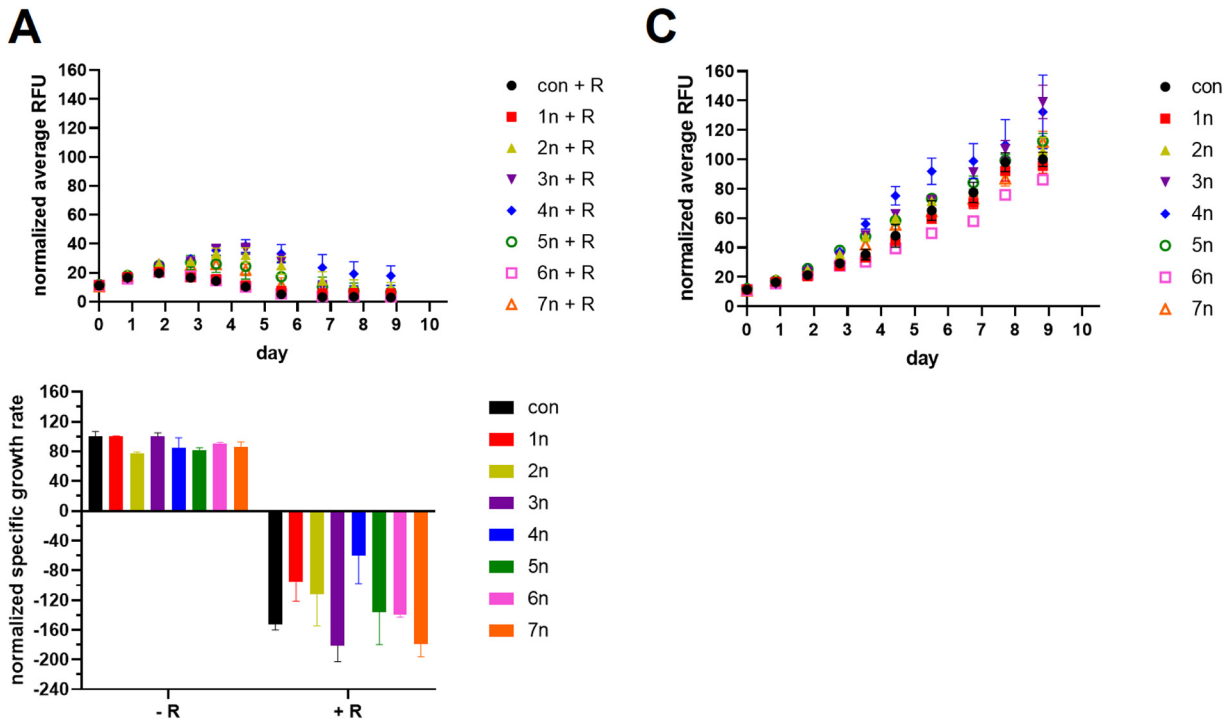

Fig. 2. Nonprotective bacterial communities (1n-7n) do not protect *M. salina* from culture crash in presence of rotifers (+R) (A), show negative specific growth rates (μ) compared to control culture (con) and co-cultures (1n-7n) without rotifers, (B), but do not inhibit *M. salina* growth in the absence of rotifers, and in some cases promote *M. salina* growth (C). All graphs show normalized replicate growth (n = 3) as measured by chlorophyll fluorescence in relative fluorescence units (RFU).

contained in ESAW) coming from microalgal carbon fixation.

Still more genera were present but below the 1% threshold for this analysis. ASVs within these lower-abundance genera contributed to moderate richness and Shannon's diversity values (averages of 141 and 2.51, respectively) of the 1p-7p communities (Fig. 3C). The richness is much lower than observed in outdoor cultures [31] or marine environments [27], though this is to be expected considering the closed system and controlled conditions. Still, maintenance of numerous bacterial taxa over months of isolation (i.e., dispersal limitation) and homogenizing forces suggests that growth dynamics, multiple

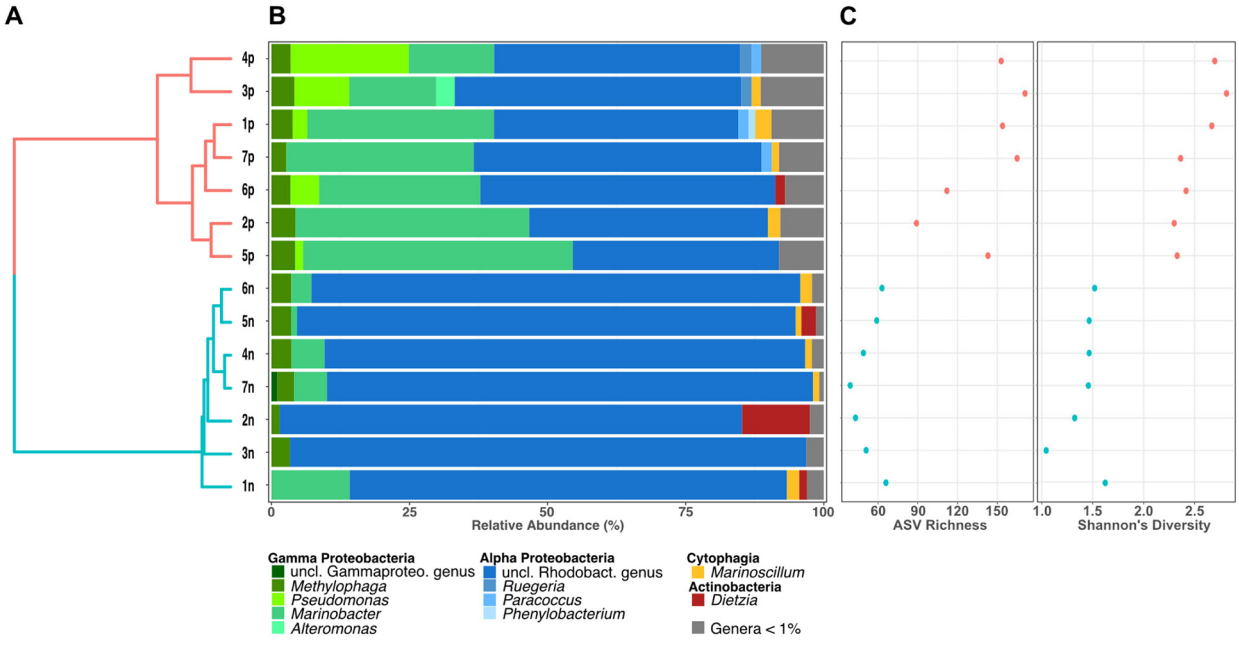

Fig. 3. Composition and diversity of bacterial consortia. (A) Hierarchical clustering dendrogram by Bray-Curtis dissimilarity reflect the compositional similarities of the bacterial communities. Red indicates 1p-7p, while blue indicates 1n-7n. (B) Taxonomic composition of the communities was determined by classification of 16S rRNA gene-based amplicon sequence variants (ASV; 100% sequence identity) using Ribosomal Database Project (RDP) rRNA sequence classifier. Taxonomy is displayed at the genus level. Genera compromising < 1% of a library's relative abundance are grouped and labeled as 'Genera < 1%'. (C) Alpha diversity of communities as ASV richness (number of detected ASVs) and Shannon's diversity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ecological/metabolic niches of an algal culture, and grazing pressure by *B. plicatilis* can preserve some degree of ecological diversity [37].

The sequencing results revealed substantial changes in the community composition of 1n-7n, where Rhodobacteraceae increased to make up 79 to 94% of the communities, while all other groups were drastically reduced to below the 1% threshold (Fig. 3B). Due to these sweeping changes, the 1n-7n communities were highly similar to each other (Fig. 3A; average Bray-Curtis dissimilarity of 0.13; 0.04–0.22 range) and shared little resemblance to 1p-7p cultures (Bray-Curtis dissimilarity range of 0.49–0.69; 0.60 average). *Marinobacter* decreased in all nonprotective communities to 0.6–14%. *Pseudomonas* was absent from all nonprotective communities, though it was inconsistent in protective communities. *Methylophaga* was mostly still present at approximately the same percentage (2.7% average) but dropped to 0.6% in 1n. *Dietzia* and *Marinoscillum* were also present, though inconsistent in 1n-7n. Further, all *Ruegeria*, *Paracoccus*, *Phenylobacterium*, and *Alteromonas* dropped below the 1% threshold for all nonprotective communities (Fig. 3). The low-abundance genera ('Genera < 1%') also decreased in 1n-7n, which likely explains the drop in ASV richness from 89 to 171 ASVs in 1p-7p down to 39–66 ASVs in 1n-7n. This drop in richness in combination with increasing Rhodobacteraceae abundances contributed to community unevenness and decreases in Shannon's diversity (1.04–1.62).

It seems likely that these large compositional changes, between the protective and nonprotective communities described here, are involved in the loss of protective phenotype. These community changes occurred during the 75-day period during which the algal-bacterial co-cultures 1–7 were grown in the absence of rotifers. This relaxation of a grazing-imposed selective pressure [38] seems to have allowed the opportunistic Rhodobacteraceae to dominate the culture [39] and overwhelm the other potentially protective bacteria previously selected for in earlier enrichment stages. It is important to note that our use of chloroplast-excluding primers may have biased our results to a limited degree. While it was necessary in order to avoid *M. salina* chloroplast sequences from overwhelming other 16S rRNA gene sequences, this may have also excluded contaminating picoeukaryotic microalgae and cyanobacteria, as well as potentially some bacterial groups, such as Planctomycetes, which in silico primer analysis (Probe Match, RDP) indicated may poorly amplify.

3.4. Specific bacterial community members correlate with algal protection

To dig deeper into which bacterial members may be responsible for the protective phenotype, we examined the relative abundances of ASVs and their correlations with protection, as the ASV more closely reflects bacterial strain-level identity compared to higher taxonomic levels [23]. Community similarities within protective and nonprotective groups generated nearly identical hierarchical clustering, with slight differences attributed to the inclusion of only the 15 most abundant ASVs (Fig. 4). Given the dominance of Rhodobacteraceae in all communities, it is not surprising that four of the top six ASVs belonged to this family. Three of these ASVs (ASV 1, 5 and 6) all increased from protective to nonprotective communities though in inconsistent proportions, indicating that these Rhodobacteraceae variants were distinct bacterial strains rather than multiple variant copies of ribosomal rRNA gene within a single strain (Roseobacter rRNA operon copy numbers range from 1 to 4; 24). In contrast, Rhodobacteraceae ASV 4 decreased in 1n-7n, indicating that it is a distinct strain with a differential response to culture conditions. ASV 2 and 3 (*Marinobacter*), which also had comparable initial abundances as ASV 1 in 1p-7p, decreased as well. In line with the drastic increase in ASV 1, 5, and 6 relative abundances and the previously described drop in ASV richness, the relative abundances of nearly all the remaining ASVs (with the exception of inconsistent ASV 9) also decreased in 1n-7n. This is also true for ASV 14 (*Paracoccus*) and ASV 15 (*Ruegeria*), which disappeared in nonprotective communities.

We sought to refine our understanding of which specific ASVs may be associated with grazing protection. Towards this aim, we tested for correlations between ASV abundance and protection, in terms of relative growth rate in the presence of rotifers (Figs. 1C & 2C). ASVs 3 (Rhodobacteraceae), 2 and 4 (both *Marinobacter*), which were highly abundant in 1p-7p, were positively correlated with algal protection ($p < 0.01$; Fig. 4B), which may be expected given their drastic reduction in abundance between protective and nonprotective communities. The less abundant ASVs 14 (*Paracoccus*), 15 (*Ruegeria*), 7 and 12 (both *Pseudomonas*) were also positively correlated with algal protection ($p < 0.01$) and exhibited decreases in abundance between 1p-7p and 1n-7n (Fig. 4B). It is interesting to note that 1p and 7p, two of the most protective communities (Fig. 1), both had comparatively high abundances of ASV 14 relative to other protective communities (Fig. 4A). Additionally, ASV 8 and ASV 11 (*Methylophaga*) were positively correlated with each other, but neither were positively or negatively associated with algal protection (Fig. 4B). ASV 8 and ASV 11 were two of the only taxa that were present in roughly the same relative abundance in both the protective and nonprotective communities (Fig. 4A). In contrast, ASV 1, 5 and 6 (Rhodobacteraceae) were all negatively correlated ($p < 0.01$) with protection. Improved resolution of putatively protective bacteria will benefit our future efforts to simplify the complex bacterial communities or isolate individual strains.

We focused our analysis on the 15 most abundant ASVs, which made up 83–92% of protective communities and 95–97% of nonprotective communities. We contend that high to moderate abundances of ASVs would most likely be necessary to mediate the high degree of protection we observed. However, we caution that these results may have limited value since statistics involving low counts are particularly susceptible to spurious associations [40] and are often inflated by community-wide changes that reduce relative abundances of many ASVs.

Although the strong positive correlations are a promising indication of which ASVs may have been involved in the protective phenotype, we readily acknowledge that the results of this analysis can neither definitively determine the correlated ASVs as causal agents nor discount the roles of other ASVs that were not found to be correlated. Further, by grouping the bacterial communities to achieve statistical foundation, we have influenced our analysis by assuming a common trend between communities. It has not been established that 1p-7p share a common protective mechanism, but rather multiple, potentially interactive mechanisms could be at work in the protective communities.

To address these concerns, we attempted to determine which correlated bacterial taxa are most likely to be necessary and sufficient for algal protection from rotifer grazing, by examining how the differential abundances of key ASVs are connected with varying degrees of algal protection within protective communities. We compared 2p, exhibiting the lowest algal growth rate in the presence of rotifers (40%), with other protective communities that exhibited stronger levels of protection (49–82% compared to untreated at 11.7%; Fig. 1). Despite the drastic differences in levels of protection by 2p and the strongly protective 7p (Fig. 1), both exhibited < 1% abundance of *Pseudomonas* (Fig. 3B), which has two ASVs correlated with protection. Intriguingly, 7p had *Paracoccus*, another group with a correlated taxon, present in > 1% abundance, while 2p did not. Indeed, all of the protective communities with the exception of 2p had either *Pseudomonas*, *Paracoccus*, or both present at > 1% abundances. We can examine this more closely in terms of the relative abundances of ASVs (Fig. 3A). ASVs 2, 3, 4 and 15 are consistently abundant across 1p-7p and may be necessary for protection, but they were not likely to contribute to differential protection levels. On the other hand, ASVs 7, 12 and 14 are more variable across 1p-7p. While most of the protective communities did not have high levels of all three of these ASVs, and 2p had low abundances of all three, the others had at least one or two of these ASVs present in moderate abundance. For example, 7p has low abundance of ASV 7 but higher abundance of ASV 14, while in 5p ASV 14 is absent and ASVs 7

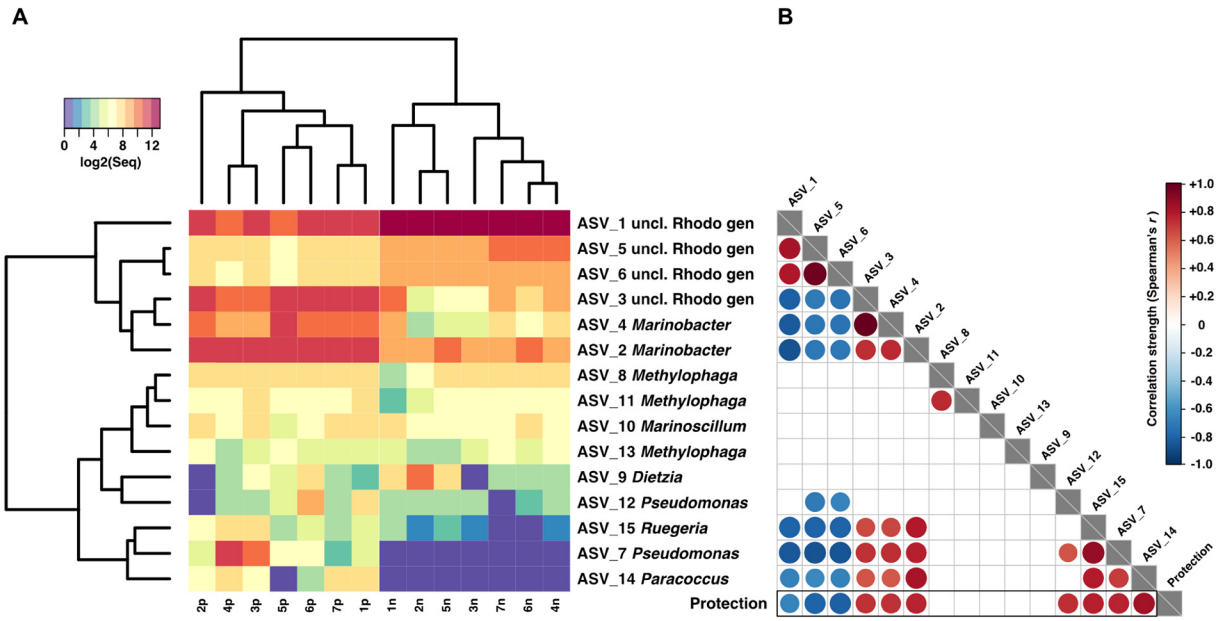

Fig. 4. Abundances of top 15 amplicon sequence variants (ASVs) and correlations with protection. (A) Heatmap displaying sequence abundances of the 15 most abundant ASVs across all bacterial communities. The order of consortia and ASVs were determined by hierarchical clustering. Color scaling reflects the number of sequences in libraries rarefied to 11,000 sequences. (B) Correlations between ASVs and specific growth rates (μ) of algal-bacterial co-cultures in the presence of rotifers from the grazing assays ('Protection'). Circle color reflects the Spearman's *r* correlation value. Only correlations with $p < 0.05$ following Benjamini-Hochberg correction are displayed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and 12 are both present. This tradeoff suggests that there may be a relationship in which *Pseudomonas* and *Paracoccus* can compensate for the other in their absence. The community with the highest level of protection, 1p, has high abundances of all three ASVs 7, 12 and 14, indicating that their protective functions may also be additive and complementary.

3.5. Putative roles of protective community members

It is well established that algal-bacterial relationships are ubiquitous and fundamental producers for marine, freshwater, and terrestrial ecosystems. This evolutionary co-dependence for survival has recently changed the paradigm for algal biotechnology to see bacterial communities as algal-culture symbionts rather than contaminants (for review, see [26,41]). With these results, we aim to further transform industrial algal production systems to include adding bacterial species and/or communities that will prevent culture crash from grazers, such as rotifers.

From our correlation analyses, we have identified ASVs from six bacterial genera that were correlated with protection and may be responsible for the protective effect observed. These taxa belonged to *Marinobacter* (ASV 2 and 4), an unclassified Rhodobacteraceae genus (ASV 3), *Paracoccus* (ASV 14), *Pseudomonas* (ASV 7 and 12) and *Ruegeria* (ASV 15). Variable, but consistent loss of these six taxonomically distinct microbes were associated with the loss of algal protection.

There was an intriguingly high number of genera with known biocidal strains that were present within protective bacterial communities. The *Pseudomonas* genus contains biocontrol agents for various terrestrial crops, as well as plant pathogens [42], which can produce numerous secondary metabolites and biocidal compounds (e.g., toxins). *Alteromonas* have been found to have algicidal activity via the release of bioactive molecules [43,44]. Strains of *Pseudomonas* and *Alteromonas* have been previously identified as phytoplankton-lytic bacteria and severely reducing algal growth [6]. Several *Paracoccus* strains are capable of producing a range of bioactive compounds, yet it is currently unknown if their toxins affect metazoa. In fact, they are most studied for their high algicidal activity [45]. The *Marinobacter* genus contains several strains that can produce biosurfactants capable of disrupting microeukaryotic and metazoan viability [46]. Further, *Marinobacter* are known for their production and release of siderophores [46,47], which have been found to be harmful to predatory microeukaryotes. Members of *Marinobacter* can also improve algal productivity [48], suggesting dual roles in protection against rotifers and algal growth promotion.

Previously, rotifer bodies have been found to contain 10–20% bacteria, of which *Pseudomonas* and *Moraxella* accounted for over 75% [49]. *Vibrio* sp. are also common in marine systems and rotifer microflora [50]. *Vibrio alginolyticus* is a typically harmless bacterium present in rotifer hatcheries; although *V. alginolyticus* Y5 has been isolated and specifically found to be especially potent to rotifer cultures in comparison to other strains of the same species [51]. Additionally, violacein-producing bacteria, such as *Janthinobacterium lividum* and *Chromobacterium violaceum*, have been found to reduce growth rates and survivorship of rotifers, *Keratella cochlearis* and *Brachionus calcyfloris* [14]. It is also possible that the bacterial community members in the algal-bacterial co-cultures were responsible for altering the metabolism, physiology, or biochemical composition of the algal culture and, thus, reducing the utility of the algae as a beneficial food source. There is precedence for bacterial species altering algal biochemical composition [52] and the dependency of rotifer culture stability on the supplied algal food source [53–55]. Further study is required to determine the mechanism of protection shown here.

Surprisingly, several of our candidate strains belong to genera known for algicidal activity. However, none of the protective communities exhibited diminished algal productivity in the absence of rotifers confirming that they were not toxic to *M. salina* (Fig. 1C). If the protective bacterial strains indeed have biocidal activity, their toxins appear to differentially affect rotifer viability and/or grazing activity with negligible impact to *M. salina*.

3.6. Reselection of protective bacterial communities

In order to re-establish protective algal-bacterial co-cultures, a frozen aliquot of 1 was grown for four weeks with untreated *M. salina*

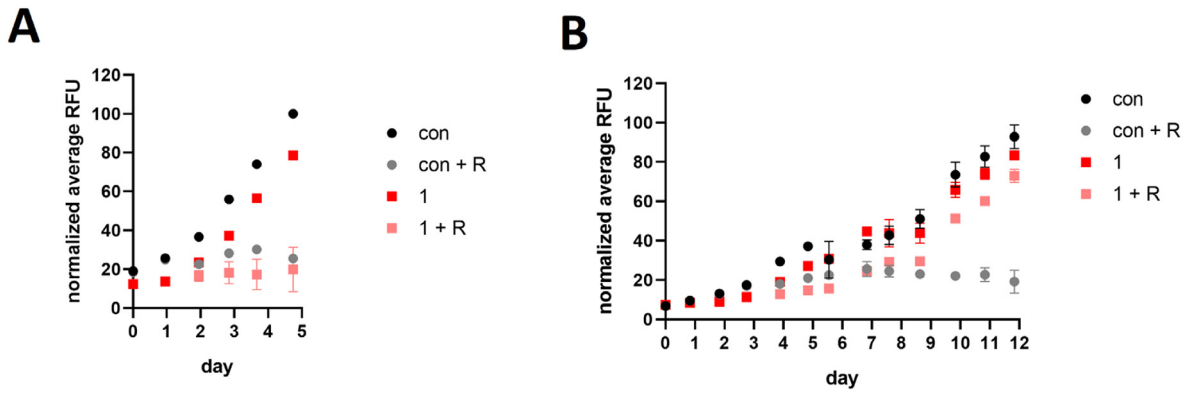

Fig. 5. Re-selection of protective bacterial community members before (A) and after (B) rotifer challenge; algal-bacterial co-culture 1 and the untreated *M. salina* control (con) are grown in the presence of rotifers (+R) or absence. All graphs show normalized replicate growth (n = 3) as measured by chlorophyll fluorescence in relative fluorescence units (RFU).

before a plate-based microalgal growth assay was conducted (Fig. 5A). Upon observation co-culture 1 was not protective, the stock algal-bacterial co-culture for 1 was then grown in the presence of rotifers to encourage the reselection of the protective bacterial community members. To better test the protection of the bacterial community in 1, the flask-based microalgal growth assay was performed (Fig. 5B). This time, the algal growth in the presence of rotifers was higher for co-culture 1 than it was for the untreated *M. salina* control (Fig. 5B).

Based on these data, our subsequent approach to culturing protective bacterial communities has incorporated routine rotifer challenges to maintain protective function. Considering the diverse, dynamic nature of the bacterial communities and potential modulation of community function (i.e., protection from rotifer grazing) with changes in culturing parameters, more focus has to be given to the ecological processes at play in structuring microbial communities. Future work in this area will determine the bacterial, chemical, and/or ecological mechanisms of this protective effect.

4. Conclusion

Our data support that inoculating production ponds with a diverse, protective microbiome may be a novel, low-cost method to reduce the frequency of pond crashes. We describe the establishment of algal-bacterial co-cultures and demonstrate their effectiveness in reducing microalgal loss to rotifer grazing. The establishment of protective algal-bacterial communities may be applicable to additional algal-grazer pairs and, therefore, may be a preferable, less expensive method than standard expensive interdiction techniques. Engineering protective algal-bacterial co-cultures to save algal ponds from crash could reduce production costs for the algal industry and drive down the costs for algal biofuel and biodiesel production.

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Author contributions

C.L.F. collected and analyzed the data and wrote the manuscript, C.S.W. analyzed the data and wrote the manuscript, J.A.K. analyzed the data and edited the manuscript, P.D.L. and T.W.L. collected and cultured the original algal-bacterial co-cultures and performed the original experiments, K.L.S. aided in statistical analysis and manuscript revisions, R.K.S., X.M., and T.W.L. contributed to analysis and interpretation of data and edited the manuscript.

Conflict of interest

The authors have no financial conflicts of interest.

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Statement of informed consent, human/animal rights

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

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