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Evaluation of phenotype stability and ecological risk of a genetically engineered alga in open pond production

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ABSTRACT

Genetically engineered (GE) algae offer the promise of producing food, fuel, and other valuable products with reduced requirements for land and fresh water. While the gains in productivity measured in GE terrestrial crops are predicted to be mirrored in GE algae, the stability of phenotypes and ecological risks posed by GE algae in large-scale outdoor cultivation remain unknown. Here, we describe the first US Environmental Protection Agency (EPA)-sanctioned experiment aimed at understanding how GE algae perform in outdoor cultivation. *Acutodesmus dimorphus* was genetically engineered by the addition of two genes, one for enhanced fatty acid biosynthesis, and one for recombinant green fluorescence protein (GFP) expression; both the genes and their associated phenotypes were maintained during fifty days of outdoor cultivation. We also observed that while the GE algae dispersed from the cultivation ponds, colonization of the trap ponds by the GE strain declined rapidly with increasing distance from the source cultivation ponds. In contrast, many species of indigenous algae were found in every trap pond within a few days of starting the experiment. When inoculated in water from five local lakes, the GE algae's effect on biodiversity, species composition, and biomass of native algae was indiscernible from those of the wild-type (wt) progenitor algae, and neither the GE nor wt algae were able to outcompete native strains. We conclude that GE algae can be successfully cultivated outdoors while maintaining GE traits, and that for the specific GE algal strain tested here they did not outcompete or adversely impact native algae populations when grown in water taken from local lakes. This study provides an initial evaluation of GE algae in outdoor cultivation and a framework to evaluate GE algae risks associated with outdoor GE algae production.

1. Introduction

Algae are an efficient photosynthetic platform that can produce food, fuel, and high value bio-products; all of which promise to be in high demand in the coming years [1]. Due to intrinsic high biomass productivities and reduced demands for arable land, algae offer an attractive alternative to plant crops for the production of many bio-products [2]. In algae, breeding and mutagenesis technologies may boost yield, while genetic engineering of specific desired traits could play a key role in enabling commercially viable yields and other desirable phenotypes [3,4]. Large-scale testing of algae for bio-products manufacturing remains in its infancy, and testing of GE algae in outdoor cultivation has not been conducted. Conversely, the benefits and risks of GE food crops have been extensively examined [5], and today millions of acres of GE crops are grown worldwide. Because outdoor production of GE algae has not yet occurred, experiments carried out under highly controlled and regulatory agency-sanctioned conditions are needed to address conceivable concerns in regard to potential invasiveness and persistence of GE algae in natural ecosystems. Likewise, there is practical need to test the stability of engineered phenotypes under outdoor cultivation conditions.

Due to their small size and enormous population numbers, micro-

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organisms such as algae can rapidly invade ecosystems in three stages: dispersal, establishment, and persistence [6,7]. While natural ecosystems may resist invasion due to biotic and abiotic factors, non-native species still have the potential to invade and alter the diversity, species composition, and/or functioning of native communities [8]. Therefore, the potential risk posed by GE algae depends on their capacity to disperse, establish themselves in wild communities, and persist in the presence of native species.

Concerns about ecological and public health impacts arising from escaped mass-cultured GE algae strains have been raised based on comparisons with invasive species [9–13], but never tested experimentally. While there are no cases of GE algae establishing in wild communities described in the literature, examples of invasive aquatic or terrestrial species with significant environmental impacts have been observed [14–17]. For example, the invasive phytoplankton *Alexandrium minutum* has been detected in algae blooms in northern Europe since 1985 [18]. Snow et al., argued that, depending upon the genetic modification, the fitness of GE algae may increase and subsequently outcompete native species. In worst-case scenarios GE algae may create harmful algal blooms, which could threaten the balance of marine ecosystems and dramatically impact resources and human health [9]. Conversely, many successful invaders have negligible, and sometimes, beneficial impacts on natural ecosystems [19]. In addition, others have argued that most commercially relevant GE traits are unlikely to confer a fitness advantage in nature, reducing the potential for environmental disruption [10]. The Snow and Henley papers arrived at very different conclusions on GE algae and neither publication was based on real world data [9,10]. So that the conversation on GE algae can move forward based on actual data and so that a responsible and informed conclusion can be reached, we conducted a small and controlled study to examine GE algae in outdoor growth experiments.

Translation of GE phenotypes from lab to field is another area of concern for commercial potential. Phenotypes that are expressed in the laboratory may prove unstable in uncontrolled environments due to loss of trait genes, mutations, interactions with native species, or altered gene expression. Whether GE organisms retain desirable traits in large-scale outdoor cultivation with variable conditions remains to be explored; to date, experts have only speculated on the potential consequences and outcomes of cultivating GE algae outdoors [9–12].

The potentially dramatic impact of invasive species on ecosystems, along with societal responses to transgenic foods, indicates that transparent and formal risk assessments of GE algae are needed to guide their development and potential future outdoor cultivation [9,20]. GE algae will need to be regulated, contained, and monitored, as their potential transformative effects on invaded ecosystems may be cryptic and difficult to reverse [21,22]. In this study we cultured a GE strain of *Acutodesmus dimorphus* in parallel with its wt progenitor in outdoor ponds, under purview of the EPA, representing the first EPA-approved outdoor GE microalgae experiment. *A. dimorphus* is a freshwater green microalgae that has a grazer-induced defense mechanism and has been identified as a potential feedstock for biofuels and biofertilizer production [23,24]. We examined the stability of two GE traits in a single strain: *Aequorea victoria* GFP expression, and increases in C14:0 fatty acid synthesis via expression of a *Cinnamomum camphora* acyl carrier protein thioesterase. We also assessed the potential of the GE strain to disperse, invade, and impact local aquatic ecosystems. Regulated field experiments such as this represent a crucial step in understanding the potential and risks of GE algae, and are essential data required to develop a regulatory process for the responsible and sustainable use of GE algae, which are key to meeting humanity's increasing need for food, fuel, and bio-products.

2. Materials and methods

2.1. Genetic modification of Acutodesmus dimorphus and approval from the EPA

2.1.1. Strains

UTEX 1237 (*A. dimorphus*) was acquired from the Culture Collection of Algae at the University of Texas at Austin and served as the wild type progenitor strain in this study. *A. dimorphus* morphology and physiology is quite similar to the species *Scenedesmus*, and the two are difficult to distinguish by visual observation alone. Both *Acutodesmus* and *Scenedesmus* are widely distributed in lakes and rivers of southern California.

2.1.2. Plasmid construction

A neutral locus in the chloroplast genome, upstream of the *psbA* gene, was chosen as the site for insertion of the trait genes. Two homology loci, (herein referred to as A3 and B3), were PCR amplified from the *A. dimorphus* chloroplast genome and PCR assembled while introducing *NotI* restriction sites to the 5′ and 3′ termini; *KpnI*, *SpeI*, *NheI*, and *XhoI* restriction enzyme sites were introduced between the two homology arms. The resulting PCR fragment was digested using *NotI* and ligated into a *NotI* digested modified pUC19. Modified pUC19 is identical to pUC19c (GenBank seq ID L09137.2) with the exception that nucleotides 187–447 (vector multiple cloning site) were replaced with a single *NotI* restriction site. Ligation resulted in the creation of the intermediate vector, p04-A3-B3. Next, the chloramphenicol acetyltransferase gene (CAT, from *E. coli*) was codon-biased for chloroplast expression, synthesized, and PCR assembled with endogenous expression elements from *A. dimorphus:* the *tufA* promoter and *psaB* terminator. During PCR assembly, *KpnI* and *SpeI* restriction enzyme sites were introduced at the 5′ and 3′ termini, respectively. The resulting PCR product was then digested with *KpnI* and *SpeI* and ligated into a *KpnI/SpeI* digested p04-A3-B3 vector, resulting in p04-793. Next, the *C. camphora* acyl carrier protein thioesterase protein sequence was codon-biased for chloroplast expression, synthesized, and PCR assembled with the *tufA* promoter and *psbA* terminator from *A. dimorphus*. During PCR assembly, *SpeI* and *NheI* restriction enzyme sites were introduced at the 5′ and 3′ termini, respectively. The resulting PCR product was digested with *SpeI* and *NheI* and ligated into *SpeI/NheI* digested p04-793, resulting in p04-838. Next, the *A. victoria* GFP amino acid sequence was codon biased for chloroplast expression, synthesized, and PCR assembled with the *psbD* promoter and *rbcL* terminator from *A. dimorphus*. During PCR assembly, *NheI* and *XhoI* restriction enzyme sites were introduced at the 5′ and 3′ termini, respectively. The resulting PCR product was digested with *NheI* and *XhoI* and ligated into *NheI/XhoI* digested p04-838, resulting in p04-863.

2.1.3. Strain construction

p04-863 was digested using *NotI* and run on an agarose gel, resulting in the separation of the vector backbone (2.4 kb) from the vector payload (7.4 kb). Once gel purified, the vector payload was transformed into *A. dimorphus*, as previously described for other algal species [25]. Chloramphenicol-resistant transformants were PCR screened for the presence of the intergeneric sequences and for homoplasmicity. Transformants confirmed by PCR were inoculated into non-selective TAP medium [26] to facilitate chloramphenicol marker excision (recombination between the *tufA* promoter direct repeats). Briefly, cells were grown to saturation and diluted back to early log-phase several times. At each dilution, single cells were sorted to 96-well microplates containing non-selective liquid TAP medium. Cultures were then assayed for the ability to grow in the presence of 25 μg/mL chloramphenicol. Cultures unable to grow on chloramphenicol were then screened by PCR and confirmed by Southern blot for the absence of the CAT gene.

2.1.4. EPA regulatory approval

Using the EPA's "1997 Points to Consider" document as a guide (https://www.epa.gov/sites/production/files/2015-08/documents/biotech_points_to_consider.pdf), Sapphire Energy, Inc. submitted a consolidated Toxic Substances Control Act (TSCA) Environmental Release Application (TERA) on August 1, 2013, which was approved on September 25, 2013. One of the five intergeneric strains described in the consolidated TERA, SE80331 (containing *C. camphora* acyl carrier protein thioesterase and *A. victoria* GFP), was selected for field testing and scaled for the outdoor experiment.

2.2. Growth and phenotype evaluation

2.2.1. Culture scale-up and stock-pond cultivation

The wt and GE *A. dimorphus* strains were cultivated as previously described [25]. Briefly, strains were scaled and grown in 20 L carboys agitated via bubbling with air containing 1% CO_2. Carboys were used to inoculate 100 L hanging polybags, which were then used to inoculate single 800 L outdoor air-lifted stock-ponds at experimental onset. Ponds were split into duplicate ponds on day 17 of cultivation. Ponds were grown in duplicate for the remainder of the experiment. As per EPA request, all ponds were covered with bird netting. During scale-up, strains were cultured in minimal medium (MHSM1) containing 25 mM $NaHCO_3$, 1.0 mM K_2HPO_4, 9.3 mM $NaNO_3$, 0.08 mM $MgSO_4{\cdot}7H_2O$, 0.07 mM $CaCl_2{\cdot}2H_2O$, and trace nutrients [27,28]. Deionized water was used for greenhouse growth. Tap water passed through activated charcoal filters was used for outdoor pond growth. Ponds were kept at a constant volume by adding filtered tap water throughout the experiment. All greenhouse and outdoor growths were performed at the University of California, San Diego Biology Field Station (GPS coordinates: 32.885575, − 117.230162).

2.2.2. Dry weight measurement of cultures

Growth was assessed by daily biomass measurement using a Microwave Dry Weight (MWDW) method. Each day, samples were collected from the ponds and dry weights were measured in triplicate. Using a Pall filter vacuum manifold and Whatman 0.2 μm filters, 25 mL of culture was applied to each filter. Filtered samples were washed three times each with 25 mL 25 mM (2 ppt) ammonium bicarbonate solution, placed in glass petri dishes and microwaved in a 1200 watt microwave for 10 min at 70% power. Pre- and post-weight measurements were taken using a 5 place analytical balance with 10 μg resolution (Mettler Toledo, Model × 5205 DU). Dry weight was calculated using the formula: [(PostFilterWeight - PreFilterWeight)/VolumeFiltered], where PreFilterWeight and PostFilterWeight are in grams, and the VolumeFiltered is in liters.

2.2.3. Algae flocculation and pond harvest

After pond dry weight measurement, the amount of algal biomass that needed to remain in the pond to produce a final density of ~0.2 g/L (after the addition of fresh media to the pond) was calculated. The removed pond volume was placed into a separate container and concentrated by flocculation via polymer addition. Polymer interacts with the cell surface charge of the algae and causes the individual cells to aggregate. At each harvest the amount of polymer necessary to flocculate the algal biomass was determined (data not shown). After polymer addition and agitation, the algal biomass flocculated and settled and was then pumped into a secondarily-contained carboy for transportation to the lab. In the lab, the flocculated algal biomass was further concentrated by centrifugation (3000 × *g* for 10 min) and processed for downstream analysis. As per the EPA's instructions. Any remaining biomass not used for downstream analysis was bleached prior to disposal.

2.2.4. Fatty acid methyl ester (FAME) content analysis

Samples were analyzed for fatty acid content using a modified version of the American Association of Analytical Chemists methods (AOAC method 991.39) [29]. Briefly, 20 mg of dried algal biomass and 0.5 mL of 0.5 M methanolic KOH were added to a glass culture tube. A solution of methyl heneicosanoate (C21:0)/Tritridecanoin (C13:0) was added as an internal standard, and samples were heated at 80 °C for 1 h. Tubes were allowed to cool, after which 0.5 mL of 10% boron trifluoride in methanol was added and samples were heated at 80 °C for an additional 30 min. After cooling samples were extracted with 1 mL of heptane and washed with 0.1 mL of saturated NaCl. Extracts were quantified by GC-FID (gas chromatography- flame ionization detection) and results reported as a percentage of ash free dry weight (AFDW).

2.2.5. GFP analysis

Images were captured on an Olympus Fluorescence microscope (Model BX51). A filter set was used (excitation 488 nm, emission 515 nm) to capture the fluorescence images. Exposures of 700 ms and 5 ms were used for fluorescence and bright field images, respectively.

2.3. Dispersal experiments

2.3.1. Dispersal trap arrangement

To examine algae dispersal potential from cultivation ponds to nearby natural water bodies, 380 L plastic tanks (dispersal traps) were placed in the four cardinal directions from the source cultivation ponds (blue circles, Fig. S1). In north and east directions, quadruplicate traps were placed at 5, 20, and 50 m from the cultivation ponds. In the west and south directions, only a single tank was placed at 5 m from the cultivation ponds due to space constraints at the field trial site. Traps were filled with 100 L tap water and supplemented with algae growth medium [30]. Prior to the outdoor experiment, a control laboratory experiment confirmed that *A. dimorphus* could grow in the supplied medium (data not shown). 50 mL samples were collected from dispersal traps three times per week and preserved at − 20 °C for qPCR analysis and metagenomic sequencing (Fig. 4 and Figs. S3-S4).

2.3.2. DNA extraction from dispersal trap samples

Samples were thawed, homogenized, and 9 mL was withdrawn from the original sample (Table S2) and placed in 15 mL tubes. Samples were centrifuged at 2200 g for 20 min (Sorvall Legend RT). The pellet was set aside while the supernatant from each sample was concentrated using an Amicon Ultra Centrifugal Filter (EMD Millipore) at 2500 g for 1 h. 0.2 mL from the top of the filter was added to the sample pellet and DNA was extracted following the PowerLyser PowerSoil DNA Isolation Kit user guide (Qiagen).

2.3.3. Sample preparation for metagenomic sequencing

Extracted DNA was amplified using primers designed to target the Internal Transcribed Spacer (ITS2) region of eukaryotic genomes. Primer sequences (5′-3′ orientation) were as follows: Forward-ACACGACGCTCTTCCGATCTgcatcgatgaagaacgcagc; Reverse: GACG TGTGCTCTTCCGATCTtcctccgcttattgatatgc. Lower case letters denote sequences used to amplify the ITS2 locus, while upper case letters represent sequences that facilitate the addition of sequencing barcodes. The ITS2 locus was amplified from each DNA sample using the Phusion High-Fidelity Kit (New England Biolabs). Samples were cycled as follows: 98 °C 0:30, 25 × (98 °C 0:10, 43–53 °C 0:30, 72 °C 0:30), 72 °C 5:00, 4 °C hold. DNA was gel purified using a QIAquick Gel Extraction Kit (Qiagen) and 1.5 μg was submitted for library preparation.

2.3.4. Library preparation, sequencing, and metagenome analysis of dispersal traps

Amplicons from the dispersal traps were indexed with standard Illumina barcodes via PCR, followed by library construction using TruSeq protocols and sequencing via MiSeq (paired end 300 bp reads).

Data was generated from 83 of the 96 samples, consistent with the efficacy of primary PCR (and with algae presence in traps). After demultiplexing, each dataset was assembled using de novo assembly in CLC Genomics Workbench (300–750 PE distance, mismatch cost: 2, insertion cost: 3, deletion cost: 3, length fraction: 0.5, similarity fraction: 0.8). Extracted consensus sequences from each contig were used to identify genus and species (where possible) via BLAST (default parameters) against the NCBI non-redundant nucleotide database (nr/nt). The species identification of the top hit was assigned a percent representation for each contig based on the number of sequence reads in that contig relative to the total sequence reads for that sample. These were then summed by genus or other classification to determine final percent representation in a given dispersal trap.

2.3.5. Pulsed amplitude modulation measurements

A photosynthesis yield analyzer, a Walz MINI-PAM, was used to assess the effective quantum yield of photochemical energy conversion in photosynthesis for the pond cultures. Like all PAM Fluorometers, it applies pulse-modulated measuring light for selective detection of chlorophyll fluorescence yield. Samples were taken daily from each pond, dark acclimated for 10–20 min and then read in triplicate. The fluorescence yield (F) and the maximal yield (Fm) are measured, the photosynthesis yield (Y = Δ F/Fm) calculated, and the data saved. This data was used as a general assessment of pond health. Generally, wt *A. dimorphus* cultures with a yield above 0.6 are considered healthy and drops in photosynthetic yield were considered indicative of culture health issues.

2.3.6. Identification of GE and wt algae migration using qPCR analysis of dispersal traps

25 μL of 10 × TE (Tris-EDTA) was added to 75 μL of homogenized dispersal tank samples and was boiled for 30 min. 0.5 μL of the lysate was added to 2.5 μL of Environmental Master Mix 2.0 (ThermoFisher), 1.75 μL H_2O, and 0.25 μL of the appropriate Taqman probe (ThermoFisher). Using the Custom Taqman Assay Design Tool, two FAM dye-labeled MGB probes were designed. The wt probe targets a 36 bp sequence deleted in the GE organism, while the GE probe targets the *psbD*-acyl carrier protein thioesterase junction. Prior to dispersal tank analysis, probes were tested for their specificity to the intended target in qPCR reactions using genomic DNA from the wt and/or GE *A. dimorphus*. Samples were cycled on a BioRad CFX384 qPCR machine using the following parameters 95 °C 10′, 40 × (95 °C 15″, 60 °C 1′, Plate Read) and analyzed using BioRad CFX Manager 3.1 Software.

2.4. Analysis of invasion potential of GE and wt algae

Wt and GE strains of *A. dimorphus* were grown in monoculture in 1 L Erlenmeyer flasks containing 750 mL WC medium in a temperature controlled incubator at 20 ± 1 °C under a photoperiod of 12:12 h of 40 ± 5 μmol photon $m^{-2} s^{-1}$ photosynthetically active radiation (PAR) [30]. The cultures were hand shaken 3 times daily to keep algae suspended and were allowed to reach stationary phase before the start of the experiment. Wt and GE *A. dimorphus* were inoculated at four different initial densities (10^3, 10^4, 10^5, and 10^6 cells/mL) in water containing wild background phytoplankton communities from five San Diego County lakes (Miramar, Murray, Poway, Santee, and Lindo). Density was measured via cell counting weekly on a DHC-N01 disposable hemocytometer (INCYTO Co. Ltd.,).

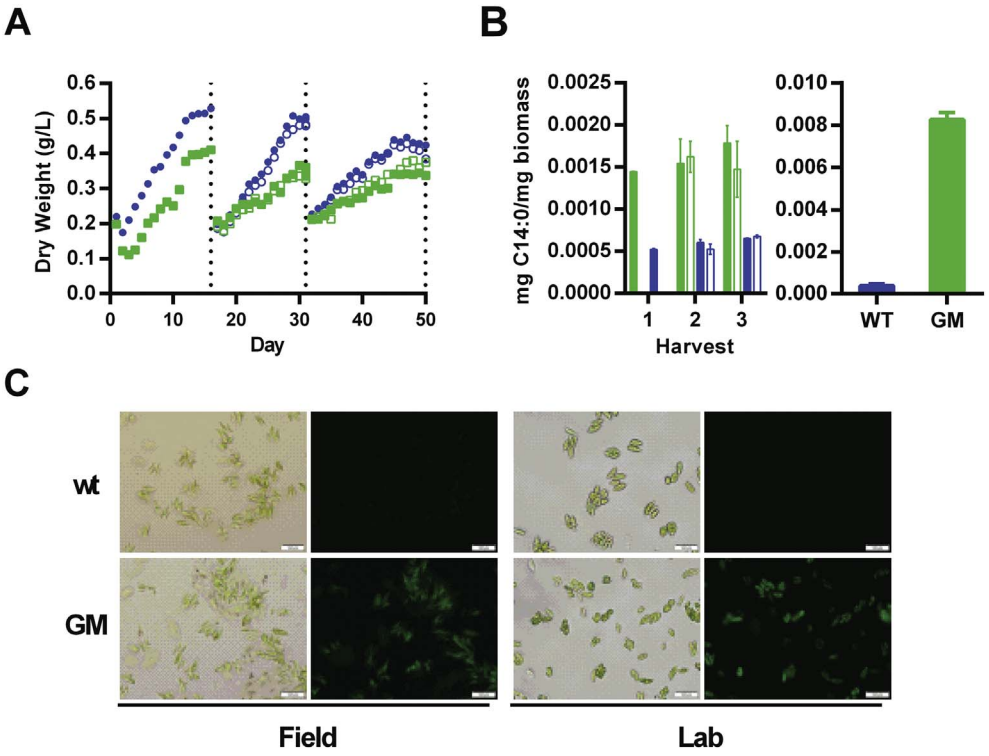

Fig. 1. Outdoor growth and phenotype evaluation of wt and GE *A. dimorphus*. (A) Cultures of wt and GE *A. dimorphus* were scaled in the University of California, San Diego Biology Field Station greenhouse and each transitioned to a single 800 L outdoor pond. Daily dry weights were collected and plotted (blue circles – wt ponds, green squares – GE ponds). At day 17 (harvest 1), wt and GE ponds were split to two separate ponds (open symbols), which were maintained for the duration of the experiment. (B) Biomass from wt and GE cultures was processed for C14:0 content by fatty acid methyl ester (FAME) conversion followed by gas chromatography for field (left) and lab (right) grown samples, normalized to mg biomass AFDW. Green bars represent GE cultures; blue bars represent wt cultures. Bar height represents mean measurement, error bars represent standard deviation of the mean. (C) Samples from wt and GE cultures collected at mid-exponential growth in the lab (3 days after initial inoculation) or field (day 10 of growth, see Fig. 1A) were analyzed by fluorescence microscopy for GFP fluorescence (scale bars = 100 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

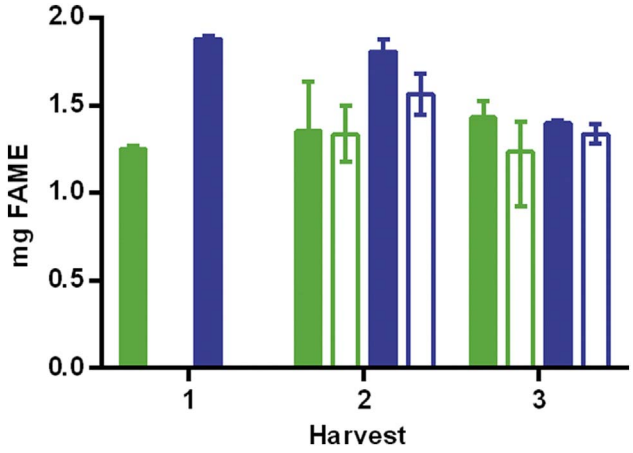

Fig. 2. Total fatty acid content of ponds. Biomass from wt and GE ponds were processed by FAME conversion followed by gas chromatography. Solid bars represent initial experimental ponds (green – GE (pond 1), blue – wt (pond 4)); open bars represent the additional ponds of wt and GE during growth periods 2 and 3 (green – GE (pond 2), blue – wt (pond 5)). Analytical replicates measured in triplicate; bar height represents mean measurement, error bars represent standard deviation of measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To examine the potential impact of wt and GE *A. dimorphus* on native ecosystems, we performed an incubation experiment inoculating the strains into intact wild communities collected in 5 gal buckets from the five local lakes on 18 October 2013. The buckets were placed inside a temperature controlled greenhouse at 25 ± 5 °C under natural sunlight. Lakes were chosen to represent a range of local freshwater environments; three oligotrophic lakes (Miramar, Murray and Poway), and two eutrophic lakes (Lindo and Santee). Four liters of water from each lake (containing resident microorganisms at ambient densities) were placed in buckets and inoculated with either wt or GE *A. dimorphus* at initial densities of 10^6 cells/mL. Control buckets containing only lake water with no *A. dimorphus* added were also maintained. Each of the three treatments (GE, wt, and control) was replicated in triplicate. In vivo chlorophyll-a measurements (proxy of phytoplankton biomass) were taken three times per week at 485 nm excitation and 685 nm emission wavelength with a Turner Trilogy Laboratory Fluorometer (Turner Designs, USA) calibrated with extracted chlorophyll-a standards. Two samples were collected on five occasions over 29 days, fixed with Lugol's iodine solution and cell counts performed as above. Impact was measured by differences in species richness (the number of wild species identified) and composition, measured by Distance Based Redundancy Analysis, dbRDA [31]. dbRDA measures similarity between experimental treatments (control, wt, and GE inoculation) in the identities and abundances of species present.

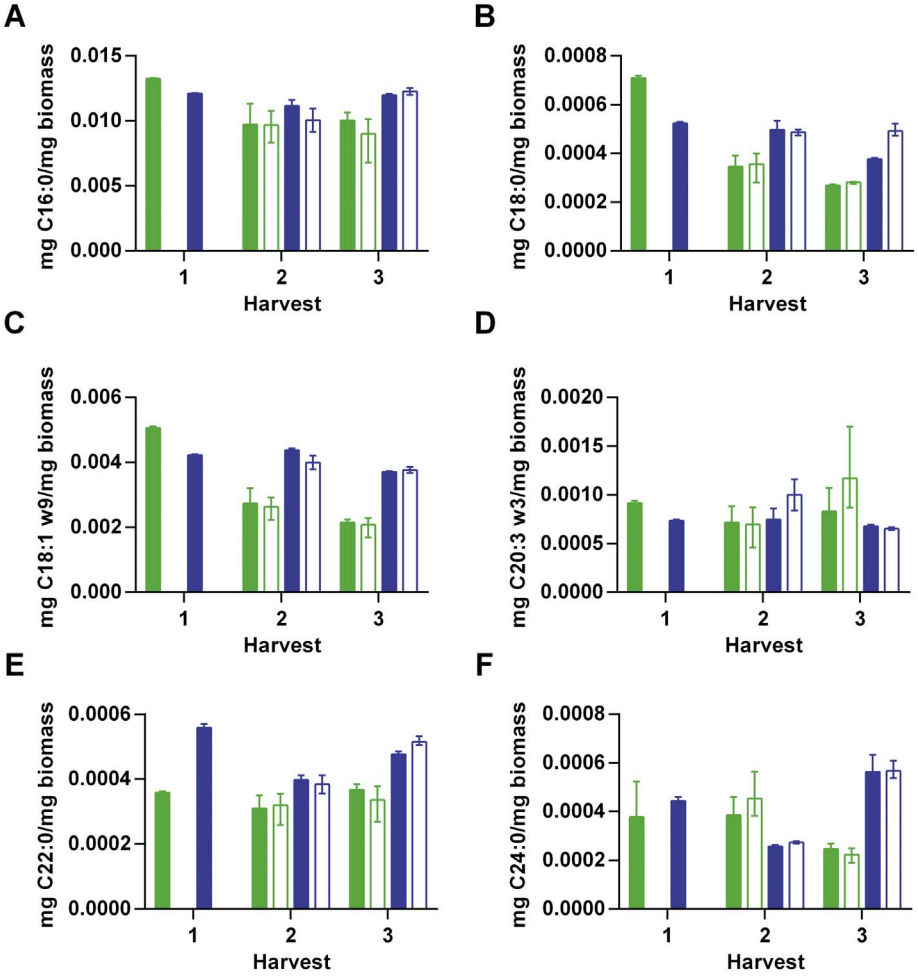

Fig. 3. Major fatty acids contributing to total pond fatty acid content. Biomass from wt and GE ponds were processed by FAME conversion followed by gas chromatography, normalized to mg biomass AFDW. Solid bars represent initial experimental ponds (green – GE (pond 1), blue – wt (pond 4)); open bars represent the additional ponds of wt and GE during growth periods 2 and 3 (green – GE (pond 2), blue – wt (pond 5)). Analytical replicates measured in triplicate; bar height represents mean measurement, error bars represent standard deviation of measurements (A) C16:0, (B) C18:0, (C) C18:1 w9, (D) C20:3 w3, (E) C22:0, (F) C24:0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

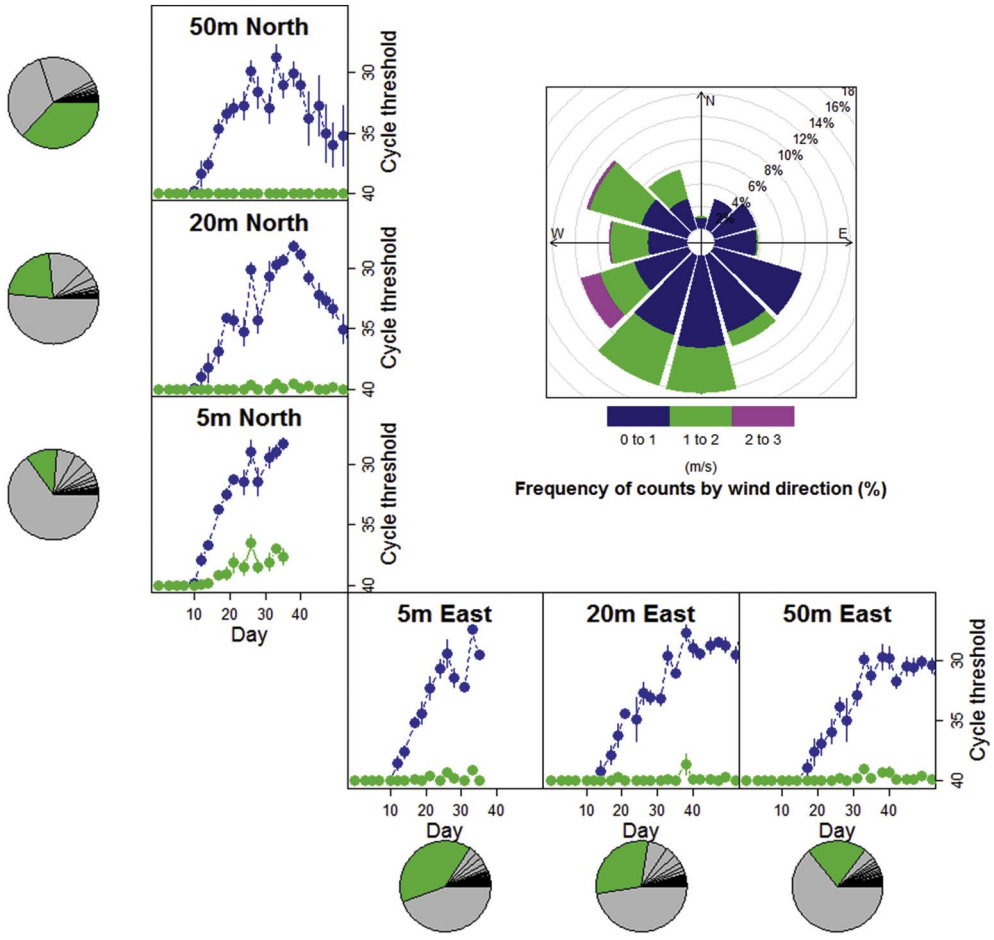

Fig. 4. Dispersal of wt and GE *A. dimorphus* grown in outdoor ponds. Samples were removed from dispersal traps three times weekly and measured by qPCR for the presence of wt or GE *A. dimorphus*. The experiment ended after 50 days except at the 5 m distance where qPCR indicated that colonization of GE occurred by day 36 (as a condition of the experimental permit, the 5 m dispersal traps were terminated at that time to mitigate further spread of the GE strain). Blue points and green points represent the average density of wt and GE *A. dimorphus*, respectively. Pie charts show the relative abundance of eukaryotic taxa identified by ITS sequencing summed over the course of the experiment, the green portion indicates *Acutodesmus* (wt and/or GE). *Acutodesmus* was the 1st or 2nd most abundant taxon found in all of the traps. The plot in the upper right shows the frequency of wind by direction (from which it is blowing) from the weather station at our field site during the experiment, with color indicating wind speed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Growth and phenotype evaluation

Two outdoor 800 L ponds were inoculated with either GE or wt *A. dimorphus* at the onset of the experiment; (Pond 1 – GE, Pond 4 – wt; see site layout Fig. S1.). Growth cycles were divided into three periods: period 1 (days 1–17), period 2 (days 18–31), and period 3 (days 32–50) (Fig. 1A). During each growth period, the cultures were grown to carrying capacity and harvested (carrying capacity is defined here as no day-over-day increase in biomass dry weight). At each harvest, a portion of the pond volume was removed and harvested by flocculation followed by centrifugation, with biomass collected for analysis. Following each harvest, fresh medium was added to reach a final culture density of ~0.2 g dry weight/L. Despite an initial drop in dry weight, correlating with low photosynthetic health (Fig. S2), the GE pond recovered and performed similarly to wt during the first growth period (Table S1). After the first harvest, ponds 1 and 4 were each used to inoculate an additional pond (ponds 2 and 5, respectively) and the four ponds were cultivated for the remainder of the experiment. All four ponds were grown to carrying capacity and harvested at the end of growth periods 2 and 3.

After growth in open ponds under ambient conditions, the GFP phenotype was easily identified in a majority of cells examined from GE pond samples (Fig. 1C, field). Additionally, C14:0 concentration, the phenotype associated with thioesterase transgene expression, was consistently and statistically ($P < 0.05$) greater in the GE strain at each of the three harvest periods (Fig. 1B). Interestingly, this increase does not correlate with increased concentrations of total fatty acids (Fig. 2), or with increased concentrations of any of the other major fatty acids analyzed (Fig. 3). The relative increase of C14:0 concentration in the GE strain was lower in the field (3-fold increase over wt) than in the lab (24-fold increase over wt) (compare left and right panels Fig. 1B). Biotic and abiotic differences between the lab and field (e.g., light penetration, temperature, fungal and bacterial contaminants, etc.) may have had a significant impact on the overall accumulation of C14:0 in the GE strain, but the observed phenotype was stable over the entire 50 days of growth. In addition, the GE strain, on average, reached lower carrying capacities than the wt in each of the three growth periods (Fig. 1A). Taken together, these results demonstrate that GE phenotypes observed in the lab can, in general, be observed in the field, though further work is necessary to optimize expression of phenotypes and growth in outdoor cultivation.

3.2. Dispersal experiments

The GE strain was detected in the traps using qPCR. The number of days to detection increased as a function of distance ($P = 0.0003$), and detection also varied with direction (distance * direction, $P = 0.004$). qPCR of the wt strain showed detectable levels in all traps by

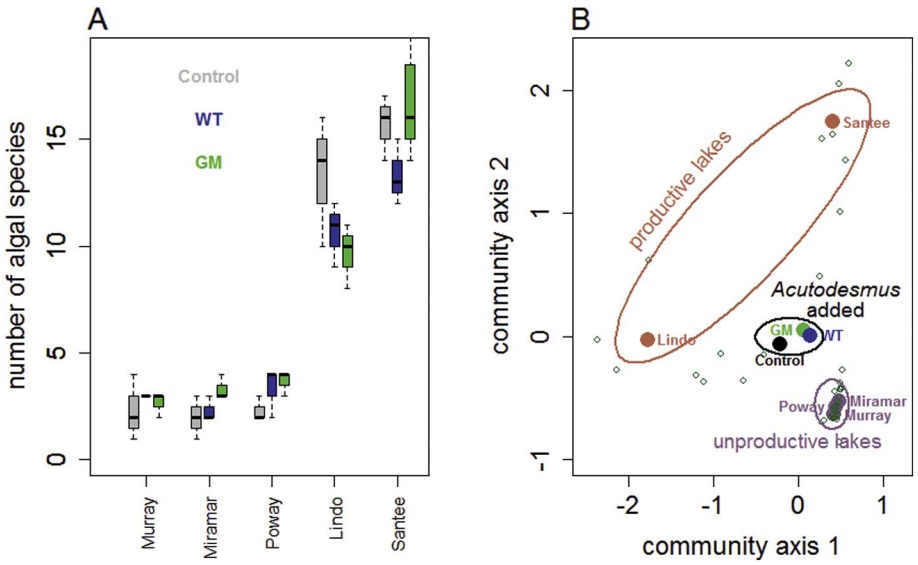

Fig. 5. Effects of inoculation with wt and GE *A. dimorphus* on native algal communities. (A) Inoculation of wt (blue bars) or GE (green bars) *A. dimorphus* into samples of local lake waters had no significant impact on native algal species richness at the end of the experiment when compared to the control. (B) Analysis of algal community composition (ordination distance based redundancy analysis) when wt (blue circle), GE (green circle), or no algae (control, black circle) were introduced into the collected lake waters. Small open points indicate individual algal communities in a water sample, and the labeled, solid symbols indicate the position of the centroids of samples taken from the same lake or experimental treatment. Position of points indicate similarity in composition such that points in close proximity represent communities with similar species present at similar densities, and more distant points indicate communities containing more distinct kinds of phytoplankton. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

10–25 days (Figs. S5–S7). At 5 m, traps were colonized by the GE strain within 15–36 days except for the tank to the west of the cultivation pond (Fig. S5), where the GE strain was never observed. At further distances, qPCR occasionally detected the GE strain at low levels except for 50 m to the north, where the GE strain was not detected (Fig. S7). The abundance of the wt strain increased throughout the experiment with a peak between 30 and 40 days, while the GE strain maintained low abundance throughout the experiment (Fig. 4 see line graphs). The consistently greater abundance of wt over GE indicates either that it has a greater propensity to disperse or that the detected wt *A. dimorphus* originated from sources other than our cultivation ponds. The latter explanation is much more likely as *A. dimorphus* is a frequent contaminant of growth ponds at the field trial site and is a common member of phytoplankton communities of local lakes.

During the experiment, algal growth occurred in all of the dispersal traps even when *A. dimorphus* (GE or wt) was not detected by qPCR. The diversity of eukaryotic organisms colonizing the traps was characterized by metagenomic sequencing using degenerate ITS2 primers to generate a PCR amplicon from DNA extracted from a subset of the dispersal trap samples (Table S2). Sequencing of the amplicons was followed by BLAST (NCBI) to identify organisms that colonized the traps over time (pie charts in Fig. 4 and Figs. S3–S4). The composition of the community within the traps fluctuated over time with no single organism dominating for > 2–3 time points. Even when *A. dimorphus* was the most abundant organism, it did not maintain dominance. *Acutodesmus* was typically one of the three most abundant genera identified by sequencing, however the qPCR data indicates that the bulk of these cells were wt and not the GE strain. Thus, a diverse community

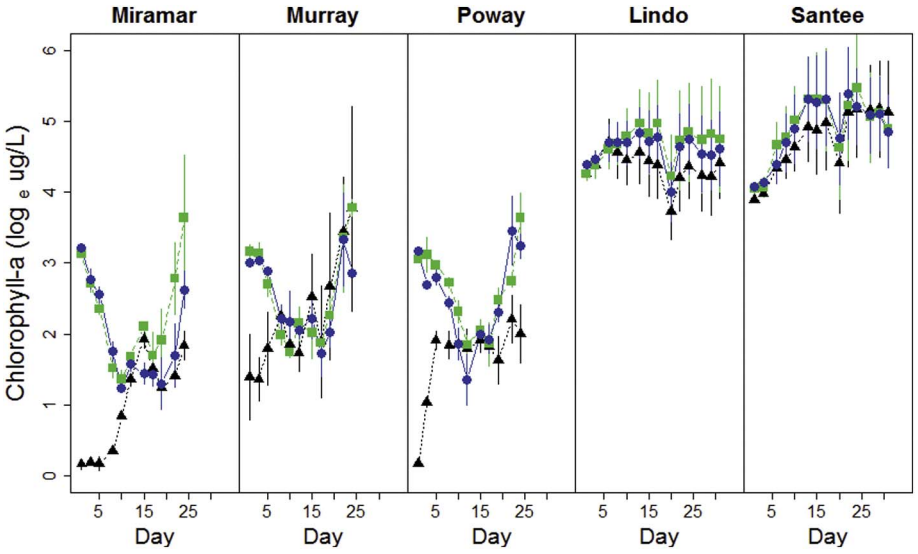

Fig. 6. Chlorophyll-a concentration of buckets during invasion experiment. Samples collected from the invasion experiment (Fig. 5) were measured for chlorophyll-a content. wt (blue circles), GE (green circles), or no *A. dimorphus* added (control, black triangles) was added to each water source in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of algal and fungal taxa, including wt and GE *A. dimorphus*, colonized the dispersal traps. That wt was more common than GE also might suggest that the genetic modification does not increase, and perhaps decreases, the organism's ability to disperse and escape the cultivation ponds, although as stated above the wt strain could have come from the local environment and not from the experimental ponds.

3.3. Invasion experiments

To test the potential impact of wt and GE *A. dimorphus* introduction on the diversity, species composition, and biomass of native species, we collected water from five local lakes each containing wild communities of phytoplankton. Based on a preliminary experiment (data not shown), we determined that *A. dimorphus* grew well when introduced at a density of 10^6 cells/mL in these waters. When inoculated at this density into the lake water samples placed in a controlled green house, there were no detectable differences between the impact of GE and wt *A. dimorphus* strains on native algal diversity (Fig. 5A), composition (Fig. 5B), or biomass accumulation (Fig. 6). Chlorophyll-a concentration (photosynthetic biomass proxy) varied among lakes, treatments, and over time (Fig. 6, all $P < 0.001$). Although there were differences among treatments and the control (no *A. dimorphus* added), there was no difference in biomass between the two treatments with *A. dimorphus* added (GE and wt, $P = 0.34$) (Fig. 5B). The effects of addition of *A. dimorphus* on algal species diversity and composition were also indistinguishable between the wt and GE strains. Fig. 5B shows an ordination of the algal communities, where points close together indicate similar species compositions. There were large differences among algal growth in waters from the different lakes, particularly between the two eutrophic lakes and the three oligotrophic lakes. However, the control, GE, and wt *A. dimorphus* treatments contained nearly identical algal assemblages at the end of the experiment, as shown by the close proximity of the GE and wt points in multivariate space. Impacts of species invasions could be different over longer time periods, and might also be different in real ecosystems. However, our results indicate that while *A. dimorphus* can invade aquatic communities that contain native species, the GE and wt algae tested behaved in a similar manner, and under our experimental conditions had no measurable impact on the diversity or composition of native algae from five lakes during the timeframe of this experiment.

4. Conclusions

The experiments reported here involve the cultivation of genetically engineered algae in outdoor field trials. These trials were conducted only after a thorough review by a panel of experts from the US EPA (via TERA submission). The results presented here illustrate an initial set of experiments for evaluating the potential benefits, challenges, and risks associated with cultivation of GE algae in open outdoor ponds. Extensive testing of algal phenotypes in an open outdoor environment will be critical since those phenotypes could vary from laboratory conditions, as they did with the thioesterase experiments described here. While the present example indicates that traits observed in a laboratory setting were expressed and stable during the entire time of these field trials, this may not be the case for every engineered trait during outdoor growth due to environmental effects on fitness or gene expression. Additionally, our ecological risk assessment indicates that while both the wt and GE *A. dimorphous* strains were able to invade traps in close physical proximity during the 50 days of our trials, neither strain was a dominant species in any trap, and neither species was able to outcompete or disrupt native populations of algae when inoculated in waters taken from five local lakes. The analysis reported here provides the basis for an informed assessment of the potential benefits and risks of GE algae during outdoor cultivation for renewable products. As the techniques and capabilities of algal genetic manipulation continue to improve, experimental observations obtained in the field must guide regulatory policies to ensure responsible use.

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Author contributions

SJS, SM, NGS, BMT, YSP, CBY, JBS, and SPM designed the experiment. SJS, SM, NGS, BMT, and SV acquired the data. SJS, SM, NGS, BMT, CBY, and JBS analyzed the data. SJS, SM, CBY, MDB, JBS, and SPM drafted and revised the manuscript. SJS, BMT, YSP, JBS, and SPM filed for and obtained regulatory permitting from the EPA.

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