

Diversity, Productivity, and Stability of an Industrial Microbial Ecosystem

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Managing ecosystems to maintain biodiversity may be one approach to ensuring their dynamic stability, productivity, and delivery of vital services. The applicability of this approach to industrial ecosystems that harness the metabolic activities of microbes has been proposed but has never been tested at relevant scales. We used a tag-sequencing approach with bacterial small subunit rRNA (16S) genes and eukaryotic internal transcribed spacer 2 (ITS2) to measuring the taxonomic composition and diversity of bacteria and eukaryotes in an open pond managed for bioenergy production by microalgae over a year. Periods of high eukaryotic diversity were associated with high and more-stable biomass productivity. In addition, bacterial diversity and eukaryotic diversity were inversely correlated over time, possibly due to their opposite responses to temperature. The results indicate that maintaining diverse communities may be essential to engineering stable and productive bioenergy ecosystems using microorganisms.

Microalgae are one of the most productive photosynthetic organisms on the planet, using sunlight to convert CO₂ and nutrients into biomass, which can be used to generate products ranging from high-value chemicals such as pigments or nutritional oils to commodities such as protein and biofuels. They can be cultivated on agricultural scales in open ponds using nonarable land and nonpotable water and as such are attractive candidates for the production of low-cost biomass (1, 2). A large limiting factor for reliable low-cost biomass production in open ponds is contamination (3–7). Managing biological contamination is costly, and while it has been achieved in open ponds for the production of high-value algal biomass (8, 9), managing algae stably in open ponds for the production of low-cost algal biomass remains challenging (10).

Agricultural pesticides or chemicals have been deployed to mitigate the challenges of contamination in algal production systems (12, 13; J. Weissman and G. Radaelli, U.S. patent application 12,321,767; R. C. McBride, C. A. Behnke, K. M. Botsch, N. A. Heaps, and C. Del Meenach, U.S. patent application 14,351,540). Approaches to managing contamination using precepts from ecology have been suggested as a viable low-cost alternative (14, 15). This perspective is informed by the idea that traits that determine fitness are not independent and often experience tradeoffs (14). For instance, Shurin et al. (16) showed that species that are good N and P competitors generally grow poorly at low light levels. Tradeoffs between other ecologically important functions have also been shown among algal taxa (17). These tradeoffs can give rise to negative associations between fitness under different conditions or abilities to perform functions such as competing for resources or resisting consumers (18–20). Tradeoffs also imply that in heterogeneous environments open to invaders, maintaining a stable monoculture will be challenging or impossible. In contrast, polycultures or ecosystems may be more stable and productive than monocultures (21). This assertion has been validated in natural and constructed algal assemblages, where increasing diversity was associated with higher productivity (22). Other ex-

periments have indicated that assemblages of algae are more efficient at taking up nutrients and resisting invasion than monocultures (14); however, more basic research is needed to determine if consortia are a viable option for algal biomass production at industrial scales. Open ponds are very distinct from the natural environments experienced by most strains of algae, where they encounter nutrient limitation, consumers and pathogens, sinking, and fluctuating environmental conditions. Whether algae in the nutrient-replete and highly productive environments of managed open ponds follow the same patterns observed in natural communities and lab experiments is still an open question.

In this study, we monitored the bacterial and eukaryotic composition of an alga pond managed to optimize biomass productivity over the course of a year. We used 16S and internal transcribed spacer 2 (ITS2) Ion Torrent Personal Genome Machine (PGM) tag sequencing to assess the bacterial and eukaryotic taxonomic composition and diversity of the pond. We simultaneously monitored a number of aspects of ecosystem structure and function (e.g., nitrate, phosphate, dry weight [DW], and fluorescence) to examine the intra- and interrelationships of ecosystem structure with genomic composition, particularly between microbial diversity and biomass productivity. We asked whether the positive relationships among diversity, stability, and productivity ob-

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served in natural and experimental communities of algae were also seen in an engineered environment managed for bioenergy production. Our study seeks to establish the applicability of ecological principles to industrial ecosystems at scales relevant to production of biomass to generate energy or specialized products. Based on ecological theory (14, 16, 22, 23), we expect that periods of high taxonomic diversity should be associated with high and more stable biomass production.

MATERIALS AND METHODS

Pond data collection. The algae were grown in a dirt-lined half-acre pond on the Las Cruces, NM, test site of Sapphire Energy Incorporated. The pond was filled with water in June 2011 and became colonized by green algae, and nutrients were added. The pond had a volume of 400,000 liters, and water was circulated via a pump at an average speed of 10 cm/s. The maximum depth of the pond was 30 cm. The pH of the pond was maintained at 9 via the addition of CO₂, and biomass was maintained between 0.4 and 0.8g/liter by harvesting (for harvest data and biomass, see Fig. S1 in the supplemental material). The medium (i.e., initial concentrations of the pond) was made up of a salt component to simulate possible commercial-level total dissolved solids (TDS) and the salt composition of water not suitable for most agricultural practices. The composition of the medium on a per liter basis is as follows: 3.675 g NaHCO₃, 4.766 g Na₂SO₄, 0.490 g KCl, 1.090 g NaCl, 0.518 g MgSO₄·7H₂O, 0.146 g NaF. The nutrient component of this medium on a per liter basis is comprised of the following: 0.3 g urea, 0.344 ml 8.5% H₃PO₄ (vol/vol), 0.06 ml trace [1 g sodium EDTA, 0.194 g ferric chloride, 0.072 g manganese chloride, 0.021 g zinc chloride, 0.013 g sodium molybdate, and 0.004 g cobalt(II) chloride into 1 liter deionized (DI) H₂O], sterilized using a Corning 0.22- μ m filter system and with 0.024 ml Fe (per liter: 336.3 g EDTA powder and 100 g Ferix-3). Ferix-3 is dry granulated ferric sulfate containing 20% Fe. Addition of nutrients such as urea, NH₄, NO₃, and PO₄ was performed to maintain the initial state of the pond medium, an N level of 100 ppm, and a PO₄ level of 40 ppm (see Fig. S2 and S3 in the supplemental material for N and PO₄ addition data, together with measured urea and PO₄ levels). The pond was treated on four separate occasions (days 152, 168, 177, and 190) with two commercial fungicides to address a decline in biomass that was suspected to be the result of fungal pathogen outbreak. The active ingredients in the fungicides applied were fluzazinam and pyraclostrobin. A 1-ppm concentration of fluzazinam was applied on days 152, 177, and 190, and 1 ppm pyraclostrobin was applied on day 168. McBride et al. (McBride et al., U.S. patent application 14,351,540) have shown the effects of fluzazinam and pyraclostrobin on uncontaminated and contaminated algae at various dosage levels, including 1 ppm, by observing the culture density (optical density at 750 nm [OD₇₅₀]). According to their study, fluzazinam has microalga toxicity at doses greater than 7.5 ppm and pyraclostrobin shows microalga toxicity at doses greater than 15 ppm. Indeed, results in the cited document demonstrate higher optical density values at applications of 1-ppm doses of fluzazinam or pyraclostrobin in contaminated algae, whereas these doses show no visible adverse effects on the optical density values in uncontaminated algae (McBride et al., U.S. patent application 14,351,540).

The pond was regularly monitored for a number of parameters, referred to as “pond ecosystem values” in this paper. Standard measurements such as temperature, pH, OD₇₅₀, OD₅₆₀, fluorescence at 430/685 nm, fluorescence at 363/685 nm, fluorescence at 590/650 nm, fluorescence at 450/685 nm, pond volume, variable/maximum fluorescence (Fv/Fm) via pulse-amplitude modulation (PAM), dry weight (grams per liter), alkalinity, NH₄, urea, NO₃, NO₂, PO₄, and harvest volume data were collected. OD and fluorescence data were collected on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). PAM measures were collected using a PAM fluorometer (Walz, Effeltrich, Germany). Alkalinity was measured on a TitroLine (Si-Analytix, Mainz, Germany), NH₄ and urea were measured using colorimetric assays (Sapphire Energy assay, similar to the assay from Seal Analytical, Mequon, WI), and NO₃, NO₂,

and PO₄ were measured using ion chromatography (IC). Dry weight was determined by standard techniques (24).

Approximately every 7 days, a biological sample was collected from the pond in a 50-ml tube. Samples were taken at a depth of around 15 cm from the same location of the pond, which was near its southwest corner. The samples were flash-frozen in liquid nitrogen within 4 h (maximum duration) of collection and stored at -80°C until processed for this evaluation. Most samples were collected within 1 h. Although the maximum duration could have skewed some prokaryotic relative abundance data, Cuthbertson et al. (25) present an acceptable window of up to 12 h without significant divergence in bacterial community, although their study suggests collection within 1 h as the optimal window, as was performed for most of our samples.

The first tag-sequencing sample used in this project corresponds to November 2011.

Sample sequencing. The PCR-amplified products of 16S and ITS2 (see “DNA preparation” in the supplemental material) were applied for bidirectional sequencing using the Ion Torrent PGM following a modified protocol for long-amplicon (400-bp) libraries using a modified long-read Ion Xpress Plus fragment library kit (Ion Torrent Community website; Life Technologies, Carlsbad, CA). Briefly, PCR products that contained a phosphate at the 5' end of each strand were directly ligated to a pair of Ion adaptors, P1 (universal) and A (barcoding), provided in the kit. The 34 samples derived from 16S gene PCR were ligated to Ion barcodes 1 to 34, respectively, while the 34 samples derived from ITS2 gene PCR were ligated to Ion barcodes 37 to 70, respectively, in a 96-well plate. The ligation was performed in a 25- μ l reaction mixture containing 50 to 100 ng PCR sample, 2 μ l ligation buffer (5 \times), 1 μ l deoxynucleoside triphosphate (dNTP) (10 mM), 1 μ l DNA ligase (5 U/ μ l), 2 μ l nick repair polymerase, 1 μ l adaptor P1, and 1 μ l of barcoding adaptor A and incubated for 15 min at 15°C and then 5 min at 72°C. After cleanup and size selection using the “magnetic bead cleanup module” (Life Technologies), the ligated samples were pooled and PCR amplified in 110 μ l of reaction mixture containing 100 μ l HiFi Platinum Taq supermix (Life Technologies), 5 μ l P1 and A primer mix, and 5 μ l of pooled samples, under an initial temperature of 95°C for 5 min followed by 8 cycles of 95°C for 15 s, 58°C for 15 s, and 70°C for 1 min. After cleanup, the PCR product was quantified by quantitative PCR (qPCR). The multiple-emulsion PCRs were performed to generate template-positive Ion Sphere particles (ISPs) following the protocol of the Ion OneTouch 2 system using the Template OT2 400 kit (Life Technologies). About 25 to 30 million template-positive ISPs were loaded onto each Ion PGM 318 chip. For 16S genes, three chips were sequenced on the PGM, while for ITS2 genes, four chips were sequenced on the PGM. The FASTQ files from Torrent Server were downloaded and used for downstream data processing.

Sequence analysis. Our ITS2 Ion Torrent PGM data contained an abundance of algal sequences. While operational taxonomic unit (OTU)-based sequence analysis approaches are widely used and provide pipelines for 16S or fungal-only ITS data in determining sample composition, they are not readily available for ITS data from other eukaryotic taxa—e.g., green algae. Grattapanche et al. (26) suggest that the cutoffs applied in OTU-based analyses are taxon dependent and that tools developed for bacterial studies (16S data analysis) are not directly applicable for all eukaryotic species (see Methods in the supplemental material for further discussion). Therefore, we mapped the 16S and ITS2 reads onto selected databases after applying certain quality controls. We also compared the results of our 16S mapping results to an OTU-based approach (see the Methods in the supplemental material) for validation purposes. We obtained Mantel test *r* statistics of 0.99, 0.98, 0.94, 0.94, and 0.91 with *P* = 0.001 for 999 repetitions for the ranks phylum, class, order, family, and genus, respectively. Diversity results from both approaches had Pearson *R* = 0.96 with *P* = 2.60 \times 10⁻¹⁴. Therefore, we confirm that the taxonomic relative abundance and diversity results in both approaches are highly similar.

In the three chips used for 16S sequencing, we obtained 1.6, 3.8, and

4.4 million reads, while the four ITS2 chips resulted in 3.7, 4.7, 4.6, and 3.7 million reads, respectively (see Fig. S4, S5, and S6 in the supplemental material for read length distributions). In order to estimate sample compositions and associated taxonomic information, we mapped our reads to the following databases: for 16S data, we used the GreenGenes 16S sequence database (version May 2013, 1.3 million sequences) (27), and for ITS2 data, we constructed a custom database from NCBI (<http://www.ncbi.nlm.nih.gov/>) using the keywords “ITS2” or “internal transcribed spacer” for sequences with a length smaller than 100,000 under the “Nucleotide” database section, which resulted in 1.1 million sequences. For mapping purposes, we used the alignment software TMAP (28), optimized to deal with variable read lengths, and Ion Torrent specific error profiles (29). See Methods in the supplemental material for detailed usage of TMAP. We filtered any read having length shorter than 50 nucleotides and an error rate higher than 2.0 for 16S reads and 4.0 for ITS reads, due to their longer average size compared to 16S reads (see Fig. S6). We accepted any mapping that breached a query coverage of 70% and a percentage of identity of 95% per hit, as applied by “16S rRNA Reference Sequence Similarity Search” by NCBI (<http://www.ncbi.nlm.nih.gov/genomes/16S/help.html#query>). (In the supplemental material, see Results and Fig. S7 and S8 for mapping statistics.) For practical purposes, among the 26,135 and 9,631 total reference sequence hits in all chips, we picked the top 2,000 and 200, corresponding to 97.16% and 96.31% of all hit reads, for 16S and ITS2 data and used their normalized hitting read counts to represent a sample composition.

We obtained the taxonomic composition of our 16S samples using the GreenGenes taxonomy, and for ITS2 samples, we used the taxonomy database of NCBI using Biopython (30). We measured sample composition similarities across all 26 genomic samples, together with the 8 technical replicates, using Bray-Curtis dissimilarity on the top 2,000 and 200 sequences’ relative abundances, for 16S and ITS2 data, respectively. We provide intrasample reproducibility assessment in Results and Fig. S9 in the supplemental material.

Here we present our results from chip 3, for both 16S and ITS2 data, due to the higher percentage of mapping reads (see Fig. S7c and S8b in the supplemental material), while simultaneously confirming reproducibility among different chips using Mantel tests (see Methods in the supplemental material), achieving r statistics in the range 0.98 to 0.99 with all other chips in both data sets.

Diversity analysis. We used Hill numbers to measure diversity with the sensitivity parameter $q = 1$, which is equal to $\exp(\text{Shannon entropy } H)$ (31–34). We computed the Shannon entropy at the genus level, after using a rarefaction of 5,000 hit reads in all samples for *Bacteria*, *Eukaryota*, *Viridiplantae*, and *Algae* diversities; and 500 for fungal diversity due to the comparably small number of hits, using 100 iterations. As shown in Fig. S11 in the supplemental material, all rarefaction curves converged. We used the functions “`rrarefy`” and “`diversity`” in package “`vegan`” (35) in R.

Diversity estimations using the top 200/2,000 reference sequences versus all hits resulted in a Pearson R of >0.99 due to the large portion (96 to 97%) of the top sequences comprised in the community (see the rarefaction curves shown in Fig. S10 in the supplemental material).

Ecosystem variables. We collected 15 pond ecosystem variables on a regular basis, ranging from every day for some variables to a few times a week for others, over the span of a year. We imputed the missing data points on a dry weight basis (grams per liter) using the OD_{750} as it had more frequent measurements and was the variable most strongly correlated with dry weight (Pearson’s $R = 0.85$). Since dry weight is the major input in the computation of productivity, and the standard deviation (SD) in productivity was of interest, imputation was a necessary step in order to have approximately similar numbers of sample sizes (see Fig. S19 in the supplemental material) in various time windows. See Fig. S20 in the supplemental material for variance patterns in original and imputed dry weight data. Other ecosystem variables than dry weight (grams per liter) were either sufficiently sampled or did not require such preprocessing as

their standard deviations were not of interest. We used the function “`mice.impute.norm.predict`” in the package “`mice`” (36) in R.

We removed the outliers (see Methods in the supplemental material) and applied linear interpolation on missing data and a 7-day central moving average smoothing. To reduce the redundancy in ecosystem variables, we identified highly positively correlated (Pearson R) groups using CAST (Cluster Affinity Search Technique) (37), with a $\theta = 0.5$, where we measured the pairwise similarities using the Pearson correlation coefficient. After finding the highly colinear ecosystem variable clusters, we standardized ($\mu = 0$, $\sigma = 1$) all variables and represented each such cluster using the first principal component of variables inside the cluster as a technique to represent/combine highly positively correlated variables (38, 39) and capture the maximum variance in the subject cluster. For example, the six ecosystem variables (average OD_{560} , average OD_{750} , DW [grams per liter], average Chloro1 fluorescence of 450/685 nm, average Green1 fluorescence of 430/685 nm, and average Cyano1 fluorescence of 383/685 nm) found in one of the ecosystem variable clusters are variables related to optical density, dry weight, and fluorescence—all sharing biological relevance to each other (see Fig. S21 in the supplemental material). Since the standardized forms of all six variables in this cluster showed similar patterns in time (i.e., have high correlation to each other), we decided to represent this cluster using their standardized first principal component, explaining 86.18% of the variance of the six ecosystem variables the cluster included.

We computed dry weight in terms of kilograms by multiplying dry weight (grams per liter) and pond volume (liter) divided by 10^3 and applying a 7-day central moving average smoothing. Finally, we obtained productivity (kilograms per day) by subtracting the two consecutive dry weight (kilogram) measurements in time with no harvesting in between. We also applied the same smoothing approach to our productivity variable. We chose to measure stability in terms of variability and used the standard deviation as the metric, following previous studies (40, 41). Thus, high stability is associated with low standard deviation in productivity over a window of days.

Ecosystem variables present a time series of data points, X_t . To reduce the variance in measurement, we computed statistics (mean and standard deviation) for ecosystem variables over a sliding window of length $2h + 1$ days (X_{t-h}, \dots, X_{t+h}), centered at each time point of the sample. The choice of window size is based on a tradeoff between reduction of measurement noise versus retention of true signal, and we used a published empirical method to identify the appropriate window size (42). Specifically, we experimented with h values of 1 to 6 weeks. The noise in mean and standard deviation patterns reduced around 3 to 4 weeks and stabilized thereafter (see Fig. S22 in the supplemental material). Moreover, the difference between two distinct peaks (days 165 and 228) in the dry weight (kilogram) data (see Fig. S12 in the supplemental material) was a duration of approximately 8 weeks. Therefore, we chose windows with $h = 4$ weeks for our figures; however, we also report final analysis results on various window sizes (see “Relationship between algal diversity and productivity measures” in the Results section).

RESULTS

Sample dissimilarity over time. The Bray-Curtis dissimilarities among all samples in the 16S data in Fig. 1a demonstrated two distinct time regions (days 1 to 100 and 200 to 350) in composition. Both time regions showed gradual increase in dissimilarity over time; however, samples in one of the distinct time regions were at roughly similar distances from all samples in the other. ITS2 data dissimilarities across all samples in Fig. 1b showed three main distinct regions in time (days 1 to 200, 200 to 300, and 300 to 350), with a fourth inner region (days 250 to 280). Sample compositions remained highly similar during the first 200 days (a dissimilarity of 0 to 0.2) and were highly different (dissimilarity of 0.8 to 0.9) around days 300 to 350, with an intermediary region of

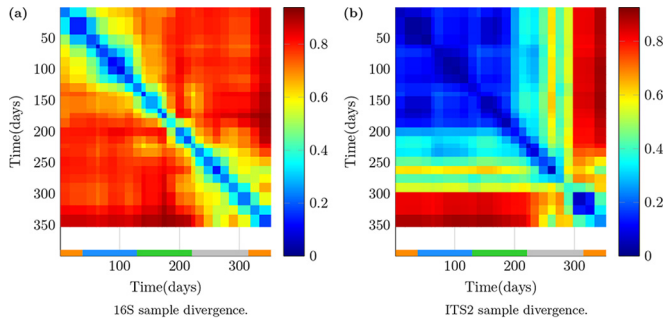


FIG 1 Sample dissimilarities. Panel a shows the Bray-Curtis dissimilarities among the samples between the bacterial (16S) samples, and panel b shows the dissimilarities between the eukaryotic (ITS2) samples. Seasons are denoted with a color bar atop the x axis as fall (orange), winter (blue), spring (green), and summer (silver).

days 200 to 300. In both bacterial (16S) and eukaryotic (ITS2) samples, we observed that sample compositions have changed the overall compositional state and showed high/increasing dissimilarity after around day 200. This roughly corresponded to the

beginning of the recovery of algal dry weight (see Fig. S12 in the supplemental material) after its sharp fall, possibly as a response to the fungicide treatment (see “Temporal bacteria and eukaryotic taxonomic profile” in the Results section for more detail).

Temporal bacterial and eukaryotic taxonomic profile. We observed the temporal taxonomic changes in the bacterial (16S) and eukaryotic (ITS2) composition of our samples using area plots for various taxonomic ranks (Fig. 2). Area plots consist of stacked relative abundances over time of taxa at different levels of taxonomic resolution. Relative abundances of less than 1% (1.3% for bacterial genera) are marked as “Other” for clarity. To examine patterns at an even finer resolution than the genus level, we also included the area plots of the top 2,000 and 200 reference sequences for 16S and ITS2 data in Fig. S13 in the supplemental material. We also placed black shading on the plots between days 152 to 190 as the duration of the four dosages of fungicide, followed by the algal dry weight recovery (day 200). We referred to the samples before day 200 as “prerecovery” and the ones after as “postrecovery.”

The phylum-level 16S composition (Fig. 2a) revealed that *Verrucomicrobia*, *Proteobacteria*, and *Cyanobacteria* comprised the

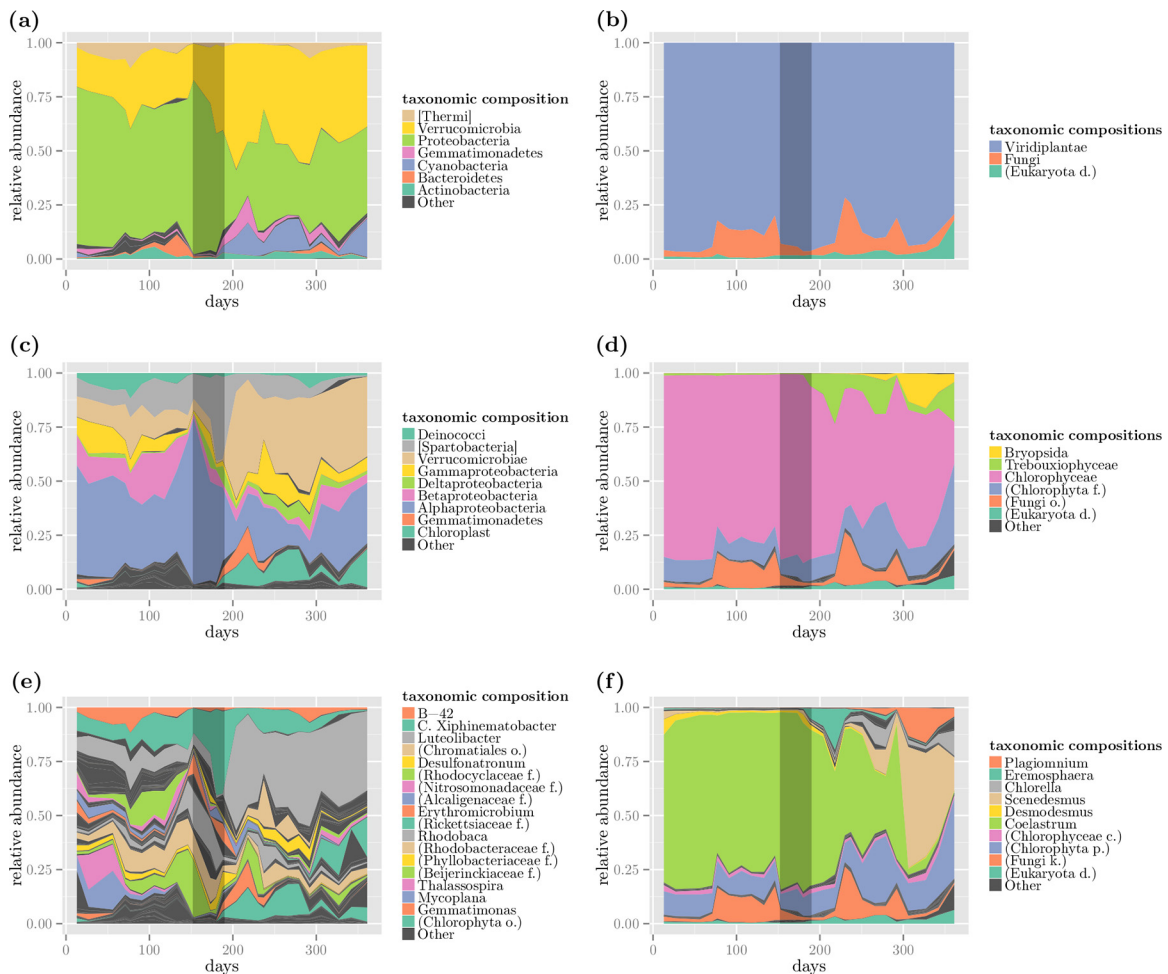


FIG 2 Area plots. The plots depict the relative abundances of various taxa and are organized with increasing level of rank in their corresponding taxonomy for 16S (left-hand side) and ITS2 (right-hand side) compositions. Plots a and b represent the relative abundances at the phylum and kingdom levels, whereas plots c and d and e and f further analyze the compositions at the class and genus levels, respectively. Taxa that had no information at their respective rank are shown in parentheses using the lowest available taxonomic rank. The black shading between days 152 and 190 represents the time interval that includes the 4 time points of fungicide application.

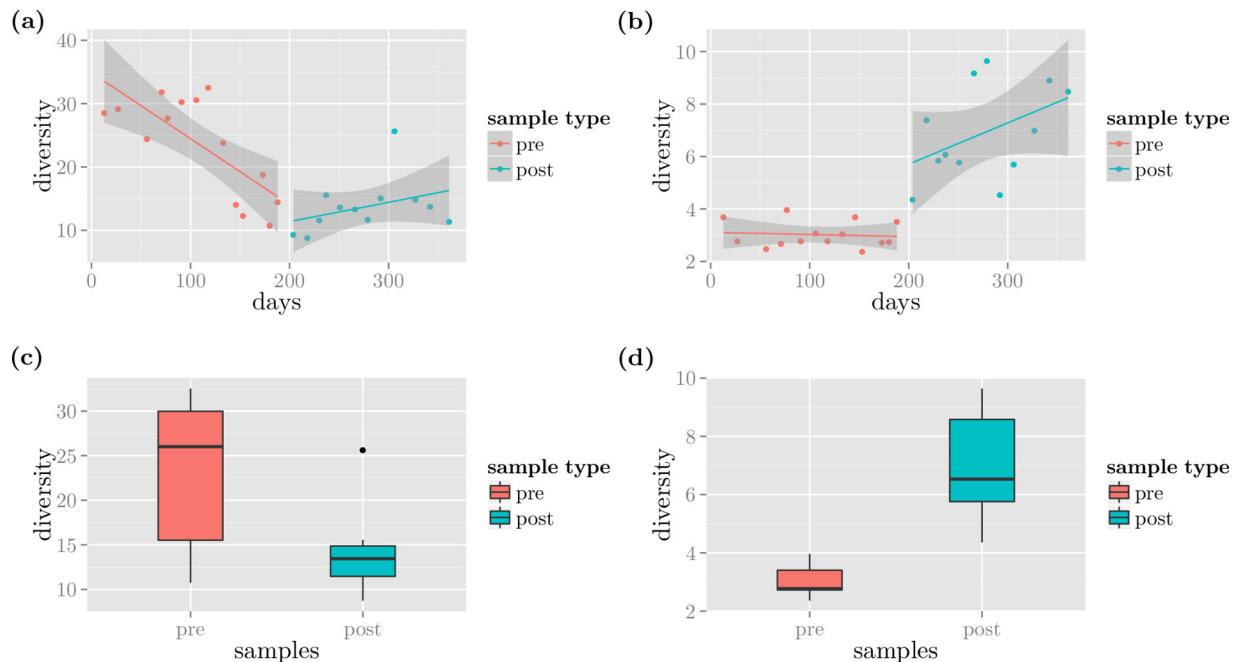


FIG 3 Diversity patterns. Panels a and b show the diversity patterns of bacterial (16S) and eukaryotic (ITS2) data, respectively, over time. Panels c (16S) and d (ITS2) show the distributions of the diversities at the two different time periods.

majority of the taxonomic profile. *Proteobacteria* decreased in the postrecovery samples, while *Verrucomicrobia* and *Cyanobacteria* increased in abundance. Analyses at the class (Fig. 2c) and genus (Fig. 2e) levels revealed that few taxa dominated the phyla present, such as the class *Alphaproteobacteria* in *Proteobacteria* and the genus *Luteolibacter* in *Verrucomicrobia*. The abundance pattern shifts in these taxa also correspond to the algal dry weight recovery, rendering *Luteolibacter* the most abundant genus in the postrecovery samples, which starting at day 204 reached a high of 48% and occupied 37% of the sample composition by day 350.

The eukaryotic (ITS2) taxonomy analysis at the kingdom level (Fig. 2b) shows that *Viridiplantae*, which mainly consist of algal species in our samples, and *Fungi* constitute the dominant community members across all time points. Although the class-level composition (Fig. 2d) was dominated by *Chlorophyceae*, the genus-level analysis (Fig. 2f) reveals striking changes in the abundance patterns of two genera: *Coalestrum* and *Scenedesmus*. The consistent dominance of *Coalestrum* changed in the postrecovery samples, followed by a sharp decline by day 300 to be overtaken by *Scenedesmus*.

The decline in the algal dry weight (kilogram) measurements (see Fig. S12 in the supplemental material) triggering the fungicide application prior to day 152 (first dosage) coincided with an increased fungal relative abundance period, whereas the time interval between days 152 and 190, where all 4 dosages have been applied, corresponds to low (lower than the overall mean) fungal relative abundances. We observed that the top five most abundant fungal sequences had high percentages of identities to *Cryptomycota*, *Chytridiomycota*, and *Amoebophilidium* sp., which were reported as algal pathogens in a previous study on a Sapphire Energy open alga pond (43). Specifically, references gi|532165669 and gi|532165968 had percentages of identity (PID) of 87%, and 89% with a *Cryptomycota* sp. strain. Reference gi|194354257 had 89%

PID with a *Chytridiomycota* sp. strain (see Fig. S16 in the supplemental material for a distance tree result), whereas references gi|532165358 and gi|532166006 had 85% identity to *Amoebophilidium* sp. strain PML-2014 isolate FD01, a sequence previously reported by Letcher et al. (43) found in Sapphire Energy ponds. All hit subject sequences were the highest-scoring BLAST hits, which contained at least a phylum-level annotation, except for gi|532165358. See Fig. S15 in the supplemental material for the sequence mapping results.

Bacterial and eukaryotic diversity over time. We measured diversity at the genus level using Hill numbers, with sensitivity parameter $q = 1$ after rarefaction to an equal number of subsamplings for all time samples (see Materials and Methods).

We detected a structural break in the temporal diversity trends around the algal dry weight recovery (day 200) in both data sets as shown in Fig. 3a and b. The bacterial diversity was high and decreasing in the prerecovery period, and it remained low in the postrecovery period, while the eukaryotic diversity showed the opposite trend.

A Chow test revealed a significant improvement in fit was achieved by modeling the data for two subintervals rather than a regression across the entire time series available ($P < 0.01$ for both 16S and ITS2 data, respectively). In addition, a two-sided Wilcoxon rank sum test showed a significant difference between the median diversities in the two different periods for both bacteria and eukaryotes, where the signal was stronger ($P = 3.05 \times 10^{-3}$ and 2.07×10^{-7} , respectively), as shown in Fig. 3c and d.

We initially observed a significant negative correlation (Pearson $R = -0.56$, $P < 0.01$) between the bacterial and eukaryotic diversities. Controlling for temperature and fungal relative abundance (suspected algal pathogen levels and the effect of fungicide on the pathogen), however, revealed that bacterial and eukaryotic diversities had no significant explanatory value for each other

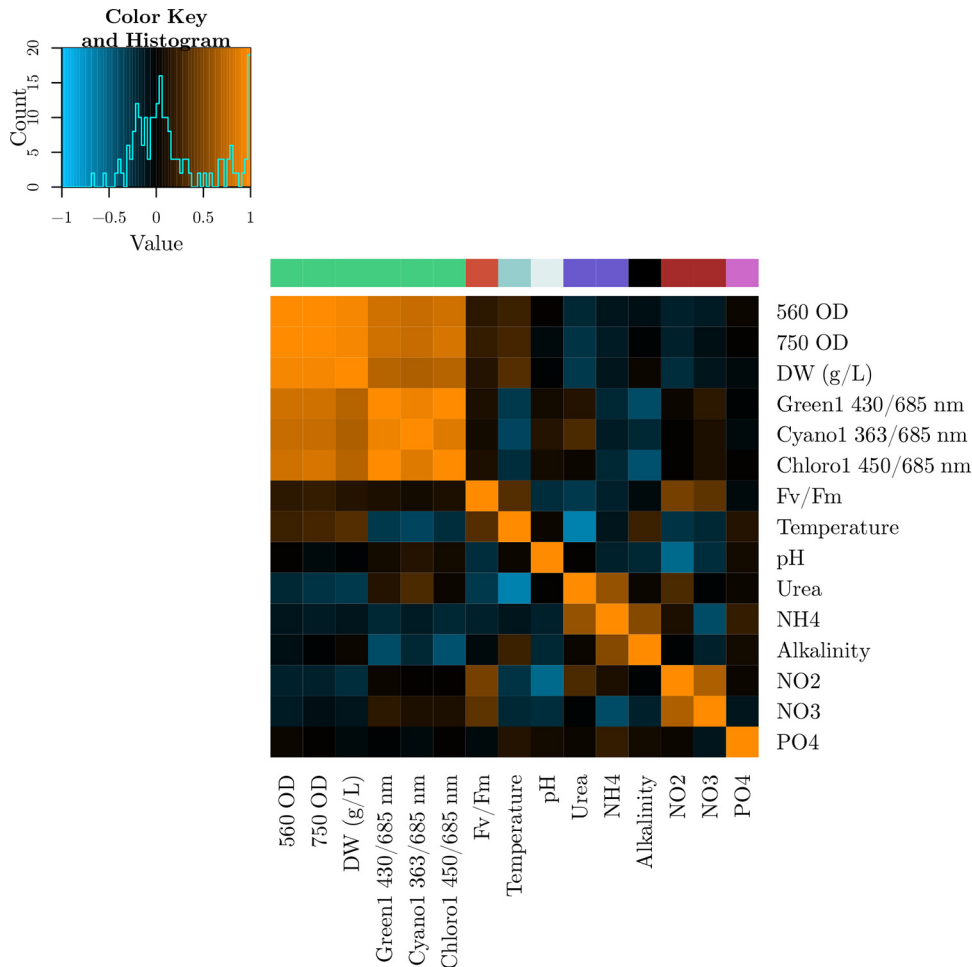


FIG 4 Correlation matrix of all phenotypic variables: Ecosystem variables forming a clique using the CAST algorithm are represented with a single color in the color bar, as also suggested by the orange correlation blocks in the matrix. Cell colors are based on the Pearson correlation coefficients according to the given color map.

($P = 0.62$). We also confirmed that fungal relative abundance did not have a significant explanatory value for bacterial or eukaryotic diversity ($P = 0.35$ and 0.57), after controlling for temperature. We, therefore, think that the initial negative correlation between bacterial and eukaryotic diversities could be due to their different responses to temperature. See section 1.7 in the Methods in the supplemental material for controlling for confounding variables and associated model comparison.

Correlations between the pond ecosystem and taxonomic composition. Although the pond was managed to maintain a stable environment through biomass harvesting and nutrient additions, we observed seasonal shifts in the availability of energy and nutrients. Figure S18 in the supplemental material shows seasonal patterns in temperature (an indicator of day length and light availability), the concentration of urea, and Fv/Fm (photosynthetic health). Urea availability peaked in winter (around days 100 and 400), while temperature peaked between days 200 and 300 (summer). Fv/Fm fluctuated strongly but showed apparent peaks in spring and fall (around days 150 and 350), with a decrease in summer, possibly due to the reduction in urea, similar to patterns in some natural phytoplankton communities (44). The sharp fall

in Fv/Fm prior to day 200 could probably be associated with the dry weight fall (see Fig. S12 in the supplemental material).

The ecosystem variables in the pond showed patterns of colinearity as well as associations with the genomic data. Figure 4 shows several variables that cluster in blocks of high correlation. We clustered the ecosystem variables using the cluster affinity search technique (CAST) (37), where pairwise similarities were measured using Pearson correlation. The 15 variables could be described by 8 independent clusters, with a θ of 0.5, which all showed the expected grouping (Table 1), including, for example, the clustering of NO_2 and NO_3 . Figure S21 in the supplemental material displays another example of an ecosystem cluster consisting of optical density, fluorescence, and dry weight measurements, alongside their standardized first principal component. Since the first principal components of all clusters explained over 75% of their variance, as shown in Table 1, the final pond ecosystem versus taxonomic composition correlations were conducted using these first principal components.

Heat maps in Fig. 5 show the Pearson correlations for kingdom diversities, and bacterial phylum relative abundances versus ecosystem clusters. Kingdom-level diversity-pond ecosystem correla-

TABLE 1 Ecosystem clusters, associated individual ecosystem variables, and percentages of variance explained by their first principal component

Cluster	Ecosystem variable(s)	% variance explained by 1st principal component
DW-fluorescence group	Avg Green1 fluorescence (430/685 nm), avg Chloro1 fluorescence (450/685 nm), avg Cyano1 fluorescence (363/685 nm), avg OD ₅₆₀ , avg OD ₇₅₀ , DW (g/liter)	86.18
Fv/Fm	Avg Fv/Fm	100
pH	pH probe	100
Temp	Temp probe	100
Urea-NH ₄ group	Urea (ppm), NH ₄ (ppm)	78.64
PO ₄	PO ₄ (ppm)	100
Alkalinity	Alkalinity (ppm)	100
NO ₂ -NO ₃ group	NO ₂ (ppm), NO ₃ (ppm)	82.83

tion analysis (Fig. 5a) showed that *Bacteria* and *Viridiplantae* had antagonistic correlations with temperature and the urea-NH₄ group. *Viridiplantae*, in addition, showed a positive correlation with the DW-fluorescence group as well. Diversity of *Fungi*, on the other hand, was positively correlated with alkalinity and urea-NH₄ and negatively with DW-fluorescence.

Temperature, pH, and the urea-NH₄ and NO₂-NO₃ groups were the major ecosystem variables to show correlation with the relative abundances of bacterial phyla, as displayed in Fig. 5b. The row dendrogram also showed that there were two major clusters of bacterial relative abundance patterns at the phylum level, based on the correlations with ecosystem variables.

Relationship between algal diversity and productivity measures. We investigated the relationship between algal diversity and the mean and standard deviation (SD) of pond productivity measurements (kilograms per day), centered at genomic sampling dates (see Materials and Methods). We removed the only non-

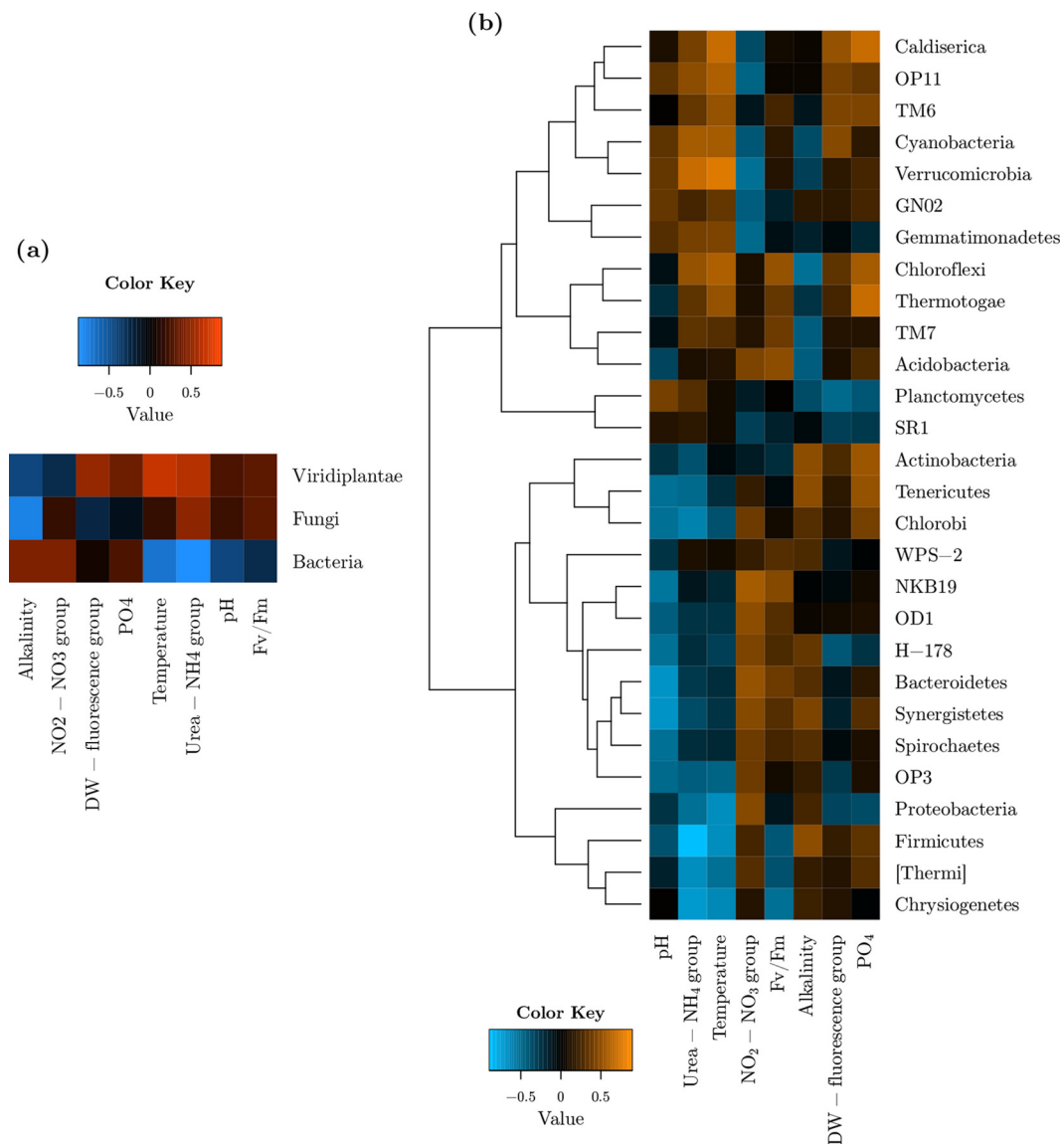


FIG 5 Pond ecosystem and taxonomic composition correlations. Panel a shows the correlations between ecosystem clusters and diversities at the kingdom level, whereas panel b shows bacterial phylum relative abundance correlations.

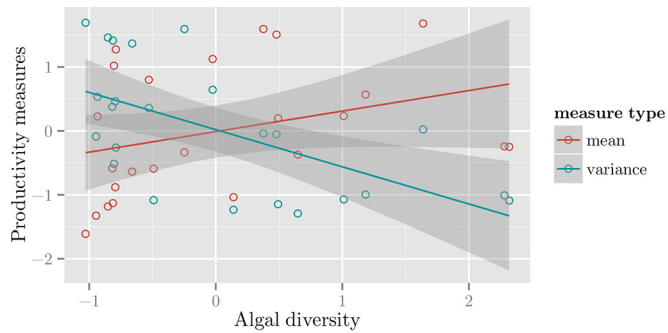


FIG 6 Correlations of the productivity mean and standard deviation versus algal diversity. The scatter plot shows the correlations between algal diversity versus the mean and standard deviation of productivity measurements centered around genomic sampling days for $2h = 8$ weeks (see Materials and Methods) using the regression lines. Algal diversity is positively correlated with mean productivity (Pearson $R = 0.33$, $P = 1.1 \times 10^{-1}$) and negatively correlated with standard deviation in productivity (Pearson $R = -0.6$, $P = 1.9 \times 10^{-3}$).

algal genus, *Plagiomnium* (class *Bryopsida* [moss]), from the *Viridiplantae* composition for calculation of algal diversity. **Figure 6** shows the relationship between algal diversity and pond productivity statistics. Algal diversity was positively correlated with the mean (Pearson $R = 0.33$, $P = 1.1 \times 10^{-1}$) and negatively correlated with the SD in productivity (Pearson $R = -0.6$, $P = 1.9 \times 10^{-3}$), suggesting high stability in biomass production.

In order to control for temperature and fungal relative abundance (suspected algal pathogen levels and the effect of fungicide on it) as potential confounding variables, we used a model comparison using the F test to examine the explanatory value/power of algal diversity for the productivity mean and standard deviation. We conducted our analysis with various window sizes (h from 16 to 36). Our results show that algal diversity has significant explanatory value for both the productivity mean and SD ($P < 0.05$) for $h = 22$ through $h = 32$ (window sizes of 45 to 65 days) and the productivity SD for $h = 34$ and $h = 36$ as well, as **Table 2** indicates. Temperature, however, did not have a significant explanatory value (when controlled for algal diversity and fungal relative abundance) for any of the window sizes experimented with (**Table 3**). Although the explanatory values of temperature for

TABLE 2 Explanatory values of algal diversity for algal productivity statistics, controlling for temperature and fungal relative abundance^a

h value	Productivity	
	Mean	SD
16	8.2e-02	9.9e-02
18	8.4e-02	7.6e-02
20	7.3e-02	5.4e-02
22	4.3e-02	4.8e-02
24	2.3e-02	4.6e-02
26	1.5e-02	4.2e-02
28	1.6e-02	1.5e-02
30	2.3e-02	1.3e-02
32	3.9e-02	1.2e-02
34	6.9e-02	9.1e-03
36	1e-01	5.4e-03

^a Results are shown for production across various h values (half window size) given as column numbers. Statistically significant explanatory values are shown in boldface.

TABLE 3 Explanatory values of temperature for algal productivity statistics, controlling for algal diversity and fungal relative abundance^a

h value	Productivity	
	Mean	SD
16	1.1e-01	5.8e-01
18	1.3e-01	5.2e-01
20	1.4e-01	4.2e-01
22	1.2e-01	3.9e-01
24	9.3e-02	4.5e-01
26	7.8e-02	5.6e-01
28	8.5e-02	8.4e-01
30	1.3e-01	8.6e-01
32	2.1e-01	8.6e-01
34	3.4e-01	7.5e-01
36	4.6e-01	6.2e-01

^a Results are shown as production across various h values (half window size) given as column numbers.

$h = 24$ through $h = 28$ had $P < 0.1$ for the productivity mean, they had $P > 0.3$ for the productivity SD in all window sizes. Since other ecosystem variables (such as urea, NH_4 , or PO_4) were highly affected by the maintaining of the nutrient supply, unlike temperature, we refrained from adding them into a predictive model.

DISCUSSION

Open alga ponds as an agricultural platform have the potential to revolutionize the production of low-cost biomass for food, fuel, and specialty chemicals if their productivity can be optimized and their stability maintained. Research into this effort has generated progress in terms of the scale, productivity, and stability of these ponds; however, substantive challenges remain. A novel and potentially transformative solution is to switch from the traditional agricultural paradigm of monocultures to one that deploys multiple strains (polycultures). The first step in this process is to understand if the benefits that have been ascribed to increased diversity in natural systems also occur in open ponds, which are very distinct from most natural systems as algae typically are maintained at a high density and not limited by any resource except for light. In this study, we observed the relationships between algal diversity and both algal productivity and the standard deviation of productivity in an open alga pond managed to maintain productivity but open to colonization from aerial sources of microbes. We found a positive relationship between productivity and algal diversity and a negative relationship between the standard deviation in productivity and algal diversity, suggesting that research into how to construct and manage consortia for deployment in open ponds may be an effective tool for pond management, as indicated by studies of natural and experimental systems (14, 16, 22, 23).

Our study reveals that managed open alga ponds for the production of biomass energy sustain a diversity of microbial life and a dynamic variability. The most common bacteria phyla observed in our study included the *Proteobacteria*, *Verrucomicrobia*, and *Cyanobacteria*, the same groups that dominate natural aquatic assemblages (45). Interestingly, the most abundant genus during the high and stable algal biomass yield period, *Luteolibacter*, under *Verrucomicrobia*, contains species that utilize algal metabolites as carbon and nutrient sources, such as

Luteolibacter yonseiensis and *Luteolibacter algae* (46, 47). Community composition also showed seasonal shifts comparable to natural assemblages (48), even though the environment was managed to achieve relative homeostasis. Our results indicate that diversity and dynamic variability are unavoidable features of open alga ponds that should be incorporated as part of their design and management.

Kingdom-level eukaryotic taxonomic composition analysis (Fig. 2b) revealed three time intervals (days 77 to 146, days 230 to 251, and around day 292) with continuous high (higher than overall mean) fungal relative abundance, with a decrease between the first and second intervals. This decreased fungal relative abundance period (days 147 to 229) encompassed the four fungicide application time points (days 152, 168, 177, and 190). Although we observed a dry weight fall soon after the first high fungal relative abundance time interval, we did not see a similar fall in biomass during or after the other two intervals. We would like to note, however, that the algal community compositions were different across the intervals. While the first time interval coincided with low algal diversity, a more diverse algal community was observed in the other two time intervals. Indeed, Smith et al. (49) and Shurin et al. (14) discuss the possibility of crop protection against disease/predation through the use of mixed-species communities. Research also shows increased associational resistance against consumers in prey alga assemblages (50) due to various possible mechanisms (51). Although our observation supports the cited findings, control experiments would be required to deduce concrete conclusions.

Disentangling the causal association between diversity and productivity is complicated as diversity can be either a driving factor or a consequence of variation in productivity (52). A positive association between pond biomass productivity and diversity of eukaryotes may reflect several underlying processes. First, a more diverse algal community may acquire abiotic resources such as different mineral nutrients (23, 53) or wavelengths of light (54) more efficiently due to niche partitioning among species. Sampling effects of random selection of high-productivity species may occur in assembled communities. Finally, the supply of resources may determine diversity, with a loss of species during pulses of high resource supply (55). However, nutrients were supplied to our community at a constant high level throughout the course of the study, and biomass was maintained by harvesting. Alternatively diversity may not be the ultimate cause of high productivity or stability but rather may be an associated variable, for unknown reasons. However, our results agree with studies of natural systems showing positive associations between ecosystem productivity and stability and the diversity of the phytoplankton community (23, 56).

Our results showed that algal diversity had significant explanatory value for the productivity mean and standard deviation, after controlling for temperature and fungal relative abundance (and the effect of fungicide on it). We acknowledge that the effect of algal diversity on productivity and stability could be confounded by temperature and the usage of fungicide. Although controlling for temperature is simple, we believe that controlling for the possible confounding effect of fungicide is harder because it is a merely 4-time-point application. Therefore, we chose to use fungal relative abundance as an extra covariate, given the microalga toxicity values shown in the patent application by McBride

et al. (7.5 and 15 ppm), which were higher than the doses used (1 ppm) (McBride et al., U.S. patent application 14,351,540).

Our observations indicate that fungal pathogens may place strong limitations on the productivity and composition of algal biofuel assemblages. These results agree well with data from other algal bioenergy studies (14, 57) and natural freshwater ecosystems (58). Fungal pathogens have been shown to be important in terminating blooms of diatoms (59, 60); however, their role in maintaining productivity is not well known. Our results indicate that fungi may impose top-down control of productivity similar in magnitude to that of mesozooplankton grazers like crustaceans and may therefore shape algal community composition.

Associations between diversity and ecosystem function varied among kingdoms. While we observed a negative correlation between temperature and bacterial diversity, eukaryotic (mostly green algae) diversity showed a positive correlation with temperature. Indeed, Stomp et al. suggest a positive association between temperature and phytoplankton richness (61). It has also been reported that many green alga genera we observed in our samples and *Cyanobacteria* have optima at higher temperatures, which correspond to the higher spring/summer temperatures at our research site (62). The bacterial phylum *Verrucomicrobia* has been shown to be positively correlated with temperature (63) and to include genera (e.g., *Luteolibacter*) that have potential associations with *Cyanobacteria* (48). Our data show increased *Luteolibacter* relative abundance during periods of increased *Cyanobacteria* relative abundance and temperature (post-day 200 in Fig. 2e), which have led to the decrease in overall bacterial diversity during higher-temperature periods particularly due to the dominance caused by the single genus *Luteolibacter*.

The negative correlation we observed between diversity of phytoplankton and bacteria over time provides some indications of the nature of the eukaryotic and bacteria components of the ecosystem. Producers and microbes engage in a range of pathogenic and mutualistic interactions that may drive positive or negative feedback in diversity between the two groups (64). Phytoplankton and bacterial communities show synchronous dynamics in nature, indicating that bacterial taxa are engaged in specific interactions with phytoplankton taxa (65, 66). Our data indicate that conditions favoring high phytoplankton diversity and productivity are accompanied by low bacterial diversity. The causal basis for this association is unknown; however, the correlation could be explained by an opposite response to temperature, since bacterial diversity had no explanatory power for eukaryotic diversity, after controlling for temperature. As discussed previously, the relative abundance increases in *Luteolibacter* and *Cyanobacteria* during higher temperatures, patterns also observed by Litchman et al. (62) and Woodhouse et al. (48), could have been the main reasons for diversity loss in bacteria at higher temperatures. Alongside rising temperature, continuous invasion by airborne propagules of microalgae during a high-light-availability period could be another possible reason for increased eukaryotic diversity in the post-algal dry weight recovery period (67). The data therefore give no indication of a causal association between diversity of prokaryotes and eukaryotes.

Managing consortia using traditional tools such as pesticide application could be challenging for consortium stability. The data we collected showed a dramatic impact of pesticide (fungicide) application on the fungal relative abundance and the

recovery of algal dry weight. As mentioned earlier, our data do not allow us to discriminate among several possible causal relationships for this pattern. That said, the fungicide application may have reduced the fitness of the target algae and provided an opportunity for other competing green alga species to begin to enter, thus increasing diversity. Other traditional management tools for open alga ponds may similarly impact consortia in unintended ways. For example, some ponds are harvested using dissolved air flotation (DAF) technology, which is commonly used in wastewater treatment. This technology relies on the deployment of a polymer that binds to and aggregates algae based on the surface charge of that algae. The aggregates are then floated to the surface of a DAF tank and skimmed off for further concentration. Without accounting for differential selectivity of this approach on a consortium of algae, harvesting using this strategy would undoubtedly also impact the makeup and stability of a deployed consortium.

Our results indicate that ecological principles relating ecosystem productivity to community diversity are applicable to industrial ecosystems for the cultivation of photosynthetic microbes. Intensifying biomass yield and fostering resilience against the vagaries of the environment or contaminating organisms are keys to commercializing the industrial growth of microbial products (14, 15, 67, 68). Most research efforts in this area involve understanding the genetic basis for phenotypic traits related to production of specific compounds (69). Ecological engineering for productivity and stability has been proposed and discussed (22) but never demonstrated beyond the laboratory scale. Many ecological processes are highly scale and context dependent (70); therefore, principles demonstrated in tightly controlled laboratory studies must be validated on a whole-system scale under natural regimens of environmental variation in order to ascertain their applicability. Our study indicates that managing microbial polycultures for productivity and stability may form the basis of a viable industrial practice to advance the commercial potential of phytoplankton for bioenergy or other higher-value products.

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Three of the authors of this paper are employees of Sapphire Energy, Inc., a for-profit company, the goal of which is to commercialize the production of a renewable source of energy using algae as a production platform. V.B. holds an equity position in Digital Proteomics, LLC, a company that licenses and sells UCSD software for proteomic analysis. The terms of this arrangement have been reviewed and approved by the University of California, San Diego, in accordance with its conflict of interest policies.

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