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Title: Biodiversity improves the ecological design of sustainable biofuel systems

Running Head: Ecological design of algal biofuel systems

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Abstract

For algal biofuels to become a commercially viable and sustainable means of decreasing greenhouse gas emissions, growers are going to need to design feedstocks that achieve at least three characteristics simultaneously: attain high yields; produce high quality biomass; and remain stable through time. These three qualities have proven difficult to achieve simultaneously under the ideal conditions of the lab, much less under field conditions (e.g., outdoor culture ponds) where feedstocks are exposed to highly variable conditions and the crop is vulnerable to

invasive species, disease, and grazers. Here we show that principles from ecology can be used to improve the design of feedstocks and to optimize their potential for 'multifunctionality'. We performed a replicated experiment to test these predictions under outdoor conditions. Using 80 ponds of 1,100 L each, we tested the hypotheses that polycultures would outperform monocultures in terms of the following functions: biomass production, yield of biocrude from biomass, temporal stability, resisting population crashes, and resisting invasions by unwanted species. Overall, species richness improved stability, biocrude yield, and resistance to invasion. While this suggests that polycultures could outperform monocultures on average, invasion resistance was the only function where polycultures outperformed the best single species in the experiment. Due to tradeoffs among different functions that we measured, no species or polyculture was able to maximize all functions simultaneously. However, diversity did enhance the potential for multifunctionality – the most diverse polyculture performed more functions at higher levels than could any of the monocultures. These results are a key finding for ecological design of sustainable biofuel systems because they show that while a monoculture may be the optimal choice for maximizing short-term biomass production, polycultures can offer a more stable crop of the desired species over longer periods of time.

Introduction

In both conventional agriculture and biofuel cultivation, researchers seek to identify species with superior potential for producing food or fuel. Although many species perform well under ideal conditions, when grown at larger scales those crops are often unable to attain high biomass yields, produce biomass that is favorable for fuel production, remain stable through time despite fluctuating conditions, resist population crashes caused by disease and pests, and resist invasion by nuisance species. Successful crops must meet all of these criteria simultaneously – they need to achieve multifunctionality. Algae are a promising source of renewable biofuels, but the challenge of achieving multifunctionality has limited the commercial-scale cultivation of algal feedstocks in open ponds (Department of Energy, 2010; National Research Council, 2012). Under conditions used for mass cultivation, algae have low productivity and lipid content relative to their potential (Sheehan, Dunahay, Benemann, & Roessler, 1998; Williams & Laurens, 2010); exhibit low temporal stability and frequent crashes (Beyter et al., 2016); and are invaded by pathogens and unwanted species (McBride et al., 2014; Smith et al., 2015). Intensive

agricultural practices have not overcome the problems faced by algal feedstock cultivation (National Research Council, 2012) and would likely exacerbate environmental problems if implemented at large scales (Foley et al., 2005; Wiens, Fargione, & Hill, 2011). Given the failures of this approach, we need to develop alternative algal feedstocks that can achieve multifunctionality under the conditions used in large-scale cultivation.

A potential strategy for achieving multifunctionality would be to cultivate algae as multi-species polycultures rather than monocultures. Numerous experiments have shown that diversity increases the potential for multifunctionality by communities (Byrnes et al., 2014; Lefcheck et al., 2015). Biodiversity enhances multifunctionality when biological tradeoffs mean that no single species is capable of maximizing all of the different functions, but certain combinations of species are able to perform more functions at higher levels simultaneously than species can individually (Lefcheck et al., 2015). Although biodiversity can improve the performance of a single function compared to monocultures (Cardinale et al., 2011), multifunctionality does not require that polycultures outperform the best single species for any given function (i.e., transgressive overyielding). Thus, the effect of biodiversity on multifunctionality is distinct from the positive effects of biodiversity on productivity (Hooper et al., 2005), temporal stability (Gross et al., 2014; Hautier et al., 2015), and resistance to invasive species and pathogens (Mitchell, Tilman, & Groth, 2002; Shea & Chesson, 2002). Based on this body of evidence, numerous papers in the last decade have proposed that multi-species polycultures of algae could be used to improve several aspects of multifunctionality in biofuel cultivation, including productivity (Shurin et al., 2013), biomass characteristics (Newby et al., 2016; Stockenreiter, Graber, Haupt, & Stibor, 2011), temporal stability (Beyter et al., 2016; Nalley, Stockenreiter, & Litchman, 2014), and resisting causes of population crashes (Smith & McBride, 2015; Smith et al., 2015).

To date, there have been few tests of the hypothesis that biodiversity can improve the cultivation of algal feedstocks, and nearly all have been constrained to laboratory-scale experiments. The few laboratory experiments that have tested this hypothesis have shown that, compared to the average monoculture, diverse cultures of algae may (Liu, 2016; Shurin et al., 2013; Stockenreiter et al., 2013; Stockenreiter, Haupt, Seppälä, Tamminen, & Spilling, 2016) or may not (Narwani, Lashaway, Hietala, Savage, & Cardinale, 2016) exhibit higher total cell biovolume or lipid content, but do exhibit more stable production through time (Narwani et al.,

2016). Although laboratory experiments suggest that biodiversity could improve multifunctionality in algal biofuel feedstock cultivation, it is unknown whether those findings are applicable to conditions in the field where conditions are often less favorable.

The performance exhibited by a mono- or polyculture under laboratory conditions does not necessarily translate to outdoor cultivation. In particular, large outdoor cultures of algae exhibit sudden catastrophic population 'crashes' due to environmental fluctuations, disease, pests, and invasive species. Several studies have demonstrated the feasibility of growing algal polycultures in open outdoor ponds (Beyter et al., 2016; Bhattacharjee & Siemann, 2015; Cho et al., 2017; Sturm, Peltier, Smith, & deNoyelles, 2012), but because these studies did not simultaneously evaluate the performance for each of those same species when grown as monocultures, it is not possible to isolate the effect of species richness (as opposed to species identity) on the performance of the cultures. As a result, the hypothesis that biodiversity improves several aspects of algal biofuel cultivation remains untested under outdoor conditions.

Here we present the results of an experiment designed to test a set of hypotheses about how algal polycultures impact feedstock cultivation under field conditions. Based on predictions from the literature and evidence from ecological experiments, we aimed to test eight hypotheses in our study (see Table 1 for a summary of the hypotheses). We hypothesized that compared to monocultures, polycultures would: (H1) increase biomass production; (H2) increase the proportion of biomass than can be converted to bio-crude oil; (H3) increase temporal stability; (H4) decrease the magnitude of crash events; (H5) delay crash events; (H6) decrease the abundance of invasive algae; and (H7) delay the impact of invasive algae. For each of these hypotheses we defined quantitative measures of performance (hereafter called 'functions') and asked whether polycultures outperformed monocultures on average, whether polycultures outperformed all of their component species, and whether any polycultures outperformed the single best species used in the experiment (transgressive overyielding). Additionally, because all of these functions are important for the overall performance of a crop, we asked whether polycultures could maintain more functions at higher levels of performance than could monocultures (multifunctionality, H8). We grew four species of green microalgae as mono- and polycultures in outdoor open ponds for 10 weeks. Although polycultures did not consistently improve biomass production or stability compared to the best single species, polycultures did delay invasions by unwanted algae longer than the best monocultures could. Moreover, select

polycultures performed more functions at higher levels of performance than even the best monocultures.

Materials and methods

Species selection. The species selected for this experiment were freshwater green microalgae that 1) were part of the Department of Energy's Aquatic Species Program, 2) are widespread throughout the United States (Environmental Protection Agency, 2012), and 3) are known to contribute to enhanced biomass production (Fritschie, Cardinale, Alexandrou, & Oakley, 2014), stability (Narwani et al., 2016) and feedstock quality (Hietala et al., 2017) in our own prior laboratory experiments. Based on these prior lab-based experiments, we ranked each species and polyculture in terms of its: mean biomass concentration, mean stability of biomass through time (mean divided by standard deviation), and the mean higher heating value (HHV) of biocrude produced from hydrothermal liquefaction (HTL) of biomass. We compared all possible sets of four species (see Experimental Design) based on their overall performance (Supporting Information). Based on this ranking, we selected four species for this experiment: *Ankistrodesmus falcatus* (A), *Chlorella sorokiniana* (B), *Scenedesmus acuminatus* (D), and *Selenastrum capricornutum* (F). We employ the same species codes as our previous work for consistency (Godwin et al., 2017a; Godwin et al., 2017b; Hietala et al., 2017; Narwani et al., 2016).

Study site. The experiment was performed at the University of Michigan's Edwin S. George Reserve near Pinckney, MI, USA (42.47ºN, 84.00ºW). This reserve is situated among mixed land uses (forest, row crops, and pasture) and is predominantly covered by temperate forest and wetlands. The plot for the present experiment was located 43 m from a fenced area containing 9 large ponds (Fig. S3). The ponds are each 30 m in diameter and are separated by a border of mowed grass. The littoral vegetation is predominantly *Typha* and the ponds contained both macrophytes and phytoplankton. Prior to the experiment, we removed all vegetation from the plot and a surrounding buffer zone of 5 m. The ground was covered with permeable fabric and a layer of wood chips to stabilize the soil and prevent growth of vegetation during our study.

Experimental design. The design of the experiment included: each of the four species as monocultures, all pairs of species as 2-species polycultures, and the 4-species polyculture. As summarized in Fig. 1, each monoculture was replicated 7 times, each two-species polyculture was replicated 6 times, the four-species polyculture was replicated 8 times, and 8 units served as

controls without any inoculum of the focal species. To account for spatial effects (e.g. proximity to the ponds), we divided the plot into four spatial blocks and then used a partially balanced complete block design (Kuehl, 2000) to assign the treatments to the experimental units with every treatment replicated 1-2 times within each block. When randomizing the assignment of inoculation treatments to the experimental units, we included the constraint that adjacent ponds within blocks would not have the same treatment. The complete experimental layout is illustrated in Fig. S3.

Experimental ponds and setup. The ponds used for the experiment were circular cattle tanks made of black fiberglass-reinforced polyethylene (Fig. 1). Ponds were maintained at a depth of 50 cm, which corresponds to a volume of 1,100 L. Each pond was continuously mixed and aerated by four 30 cm air diffusers that delivered 35 L min^{-1} of air to each pond. Prior to inoculation, ponds were cleaned with high-pressure water and rinsed with concentrated hydrochloric acid to remove any mineral deposits and biofilm organisms. On the day of inoculation, each pond was scrubbed with sodium hypochlorite solution (0.33% w/v), drained, rinsed again with sodium hypochlorite solution for five minutes, and then rinsed with treated water (see Water supply and growth medium). Immediately prior to filling, the ponds were saturated with 70% ethanol and allowed to dry.

Water supply and growth medium. We used Bold-3N medium (Bold, 1949) as the growth medium in ponds because it contains high concentrations of inorganic nutrients needed to support high population densities of algae (8.82 mmoles L^{-1} nitrate and 1.76 mmoles L^{-1} phosphate) and mimics the high-nutrient conditions used for commercial production. Water for the experiment was pumped from a groundwater well located at the Reserve. This water contained a high concentration of calcium hardness (>5,000 µeq L^{-1}), which has potential to precipitate phosphate in the Bold-3N medium from solution. To avoid this problem, we removed the calcium from the groundwater using a zeolite ion-exchange resin, periodically recharged with sodium chloride. We monitored the effectiveness of this system by titration (Hach Company, kit HA-71A) and only used water with hardness below 200 µeq L^{-1}. After the softening step, water was filtered through a 10 µm woven mesh filter and disinfected using a 200 watt flow-through UV lamp (Aquaneering). Treated water was dispensed through clean hoses that were treated daily with sodium hypochlorite to prevent contamination by algae or other organisms. Ponds were filled with treated water before adding the components of Bold-3N medium.

Macronutrients, in the form of inorganic salts, were added directly to the ponds. Micronutrients were added as a single concentrated solution that had been sterilized using a 0.2 µm filter.

Inoculation process. Prior to inoculating the 1,100 L experimental ponds, we established 12 L 'inoculum cultures' at the field site. These mono- and polycultures were grown in 20 L polyethylene buckets that had been sanitized as described for the ponds. The buckets were filled with 12 L of Bold-3N medium, covered with transparent polyethylene lids, and continually aerated with air delivered via a single air diffuser. We inoculated the 12 L inoculum cultures with laboratory-grown stocks of each algal species, using a substitutive design for polycultures in which the total biomass added to each 12 L inoculum culture was constant at 1,050 mg dry mass, regardless of species richness. In the polycultures, the biomass of each species was equal to 1,050 mg divided by the species richness. The 12 L inoculum cultures were positioned adjacent to the ponds plot and were exposed to full sunlight for between 8 and 17 days. One 12 L inoculum culture was prepared for each experimental pond, including eight controls that received no algae. We sampled the 12 L inoculum cultures at the end of their incubation and detected no algae in the control units and only the appropriate species in the experimental treatments.

After sanitizing and filling each of the 1,100 L experimental ponds, we inoculated them with the entire contents of the corresponding 12 L inoculum culture. The eighty experimental ponds were established over a period of nine days. We began inoculating the ponds on 24 May, working in numeric order as shown in Fig. S3, and finished on 2 June. After inoculation, two ponds (pond 23 treatment F and pond 24 treatment DF) showed evidence of unwanted calcium phosphate precipitation, likely due to undetected hard water. Those two ponds were drained, cleaned, filled with medium, and re-inoculated on 17 June.

Sampling. The ponds were sampled via an opaque polyethylene sampling tube originating at the center of each pond and terminating outside the pond. This sampling tube was installed prior to filling the ponds and allowed for samples to be collected without a researcher having any contact with the pond, reducing the risk of any potential contamination. At the time of sampling, compressed air was injected into the bottom of each sampling tube, creating an air lift that delivered the contents of the pond into the sampling containers. Beginning on 7 June (week 1), we sampled the ponds every 7 days until 10 August (week 10). On each sampling date, we collected a total of 3.5 L from each pond. Following each sampling event, we added additional treated water to replace evaporative losses and maintain the total culture volume at

1,100 L. Due to low rainfall during the experiment the volume of the ponds never exceeded 1,100 L. The 3.5 L sample taken on each date was used to measure the following variables.

Algal biomass and species composition. We measured the biomass of algae in each pond by filtering duplicate subsamples onto dried and pre-weighed glass fiber filters (Merck-Millipore AP40, 47 mm diameter) using low vacuum pressure (<200 mm Hg). Filter samples were rinsed and dried to constant mass at 60 ºC. Filter blanks were included at the beginning and end of each sampling event. The mass change from the blanks was used to correct the mass change from the pond samples. The temporal stability of algal biomass was quantified as the inverse of the coefficient of variation (mean divided by the standard deviation) from weeks 2 through 10. Although this measure of stability represents the overall temporal variability of the culture, it does not necessarily reflect rapid changes in biomass that are characteristic of population crashes. Therefore, we defined biomass crashes as a proportional loss of biomass during a one-week period and computed the maximum crash magnitude measured in each pond. This approach allows for quantitative comparisons in terms of both the magnitude of crashes observed over a time period and the length of time prior to a crash event.

We preserved samples for algal identification and abundance by adding phosphate-buffered formaldehyde to a concentration of 1%. For each sampling date, we enumerated algae in each sample by microscopy using a hemacytometer and quantified the proportion of algal cells that were not the treatment species for that pond. Over the course of the experiment, a total of nine invader species of algae were observed in at least one sample.

Hydrothermal liquefaction. We used hydrothermal liquefaction (HTL) to convert algal biomass into biocrude, which is a precursor of renewable transportation fuels (Savage, 2012). Unlike direct lipid extraction, HTL does not require high lipid content in the algae and instead, converts whole wet biomass to biocrude (Valdez, Nelson, Wang, Lin, & Savage, 2012). We performed HTL on algal biomass samples from weeks 1 through 8. To concentrate biomass for HTL, we settled a 2.5 L sample in the dark for 7 days. The samples were further concentrated by decanting and centrifugation (Hietala et al., 2017; Narwani et al., 2016). The concentrated biomass was dried at 60 ºC until mass was constant. The full procedure for HTL follows that of our previous work (Hietala et al., 2017). In short, the dried biomass samples were mixed with deionized water to 5% solids content and subjected to HTL at 350ºC for 20 minutes. Biocrude was separated from the other products using dichloromethane and then dried under nitrogen to

247 evaporate residual solvent. For each reaction, we calculated biocrude yield as the mass of
248 biocrude product divided by the mass of algae used for HTL.
249 Data analysis – linear models. We used general linear mixed models to analyze the
250 effects of initial algal species richness and composition on: (H1) mean biomass concentration
251 (mg L^{-1}); (H2) biocrude yield (g biocrude per g dry algae); (H3) temporal stability of biomass as
252 CV^{-1} through time; (H4) maximum proportion of invader algae observed in each pond, and (H6)
253 maximum crash magnitude observed in each pond (% reduction in 7 days) For each parameter,
254 the full model consisted of the fixed effects of species richness (SR), species composition
255 (Combo, nested in SR), and week. Spatial block and pond identity were initially included as
256 random effects and retained when they significantly improved the Akaike information criterion
257 (AIC). We then removed non-significant terms stepwise until reaching the minimal adequate
258 model that contained effects of species richness, species composition, and time. Temporal
259 stability, maximum crash magnitude, and maximum proportion of invading algae are all
260 measures with only one value for each pond; thus, the effects of time and pond were not included
261 in the statistical models. Control treatment ponds were used to measure the progress of algal
262 invasions in the absence of any inoculum treatment, but were excluded from all statistical
263 analyses so that the effects of species richness and species composition were not affected by this
264 treatment. All linear model analyses were performed in R using the package lme4 (R Core Team
265 2015). When we found significant effects of species richness or species composition, we
266 performed post-hoc tests using Tukey's honest significant difference method in the R package
267 lmerTest. We used the post-hoc tests to compare the performance among levels of species
268 richness and between each polyculture and the monocultures (Table 1).
269 Data analysis – logistic models for crash and invasion timing. The proportion of ponds in
270 each treatment that exceeded the median crash magnitude (55%) on or before each date was
271 modeled using logistic regression. We also used logistic regression to analyze the proportion of
272 ponds in each treatment with at least 1% proportional representation of invading algae on or
273 before each sampling date. The logistic regressions included categorical fixed effects of species
274 richness and species composition and a continuous fixed effect of time. Logistic regressions were
275 performed in R using the function "glm". Post-hoc comparisons for logistic regressions were
276 performed as for the linear models.

Data analysis – multifunctionality. Because no single species or polyculture is likely to optimize all aspects of system performance (Godwin et al., 2017b; Hietala et al., 2017; Shurin et al., 2013), we sought to characterize tradeoffs among species and determine if polycultures can mitigate these tradeoffs if they perform more functions at higher levels of performance than monocultures do (multifunctionality, Byrnes et al., 2014; Lefcheck et al., 2015). We quantified the capacity for monocultures and polycultures to exhibit multifunctionality using a threshold approach similar to the one developed in the field of biodiversity-function (Byrnes et al., 2014; Lefcheck et al., 2015). The threshold approach compares different species compositions based on how many functions they perform at or above an arbitrary level of performance (i.e. thresholds). We used seven separate functions to describe the overall performance of each inoculation treatment: mean biomass; mean biocrude yield; mean temporal stability of biomass; mean maximum crash magnitude in 10 weeks; crash timing (based on logistic regression coefficients for ≥55% crash magnitude); mean maximum proportion of invaders in the ponds; and invasion timing (based on logistic regression coefficients for ≥1% invader algae). To allow for comparisons among various functions, we standardized performance for each function as the rank of each treatment relative to the other inoculation treatments (control ponds were excluded). Ranks were assigned such that the poorest performer was rank 1/11 and the best performer was rank 11/11 = 1. Because the number of species compositions was the same for each function, we set performance thresholds between 0 and 1 in increments of 1/11. We then tallied the number of functions that each monoculture or polyculture performed above each threshold.

The threshold approach has some known drawbacks that we sought to avoid. A recent paper showed that when the number of functions performed above a threshold is used as a dependent variable for regression, there can be artifacts that arise due to chance rather than biological effects (Gamfeldt & Roger, 2017). Thus, a 'null' model is required to detect biological effects against the background chance of an artifact. To generate a null model, we used randomization tests to assess the significance of differences in multifunctionality between mono- and polycultures at each threshold. For each performance threshold, we compared the number of functions performed by: the mean two-species polyculture and the mean monoculture, the four-species polyculture and the mean monoculture, the best two-species polyculture and the best monoculture, and the four-species polyculture and the best monoculture (Fig. S4). We then compared the observed differences to a null model based on randomized performance ranks. For

each comparison, we used the null model to generate a distribution of differences based on randomized performance ranks (n=10,000 iterations). This randomization method takes into account that there were different numbers of species compositions for monocultures, two-species polycultures, and the four-species polyculture.

Results

The original hypotheses for the experiment are summarized in Table 1. This table also provides a summary of findings from our experiment, and can serve as a reference guide for readers as we summarize all of the results.

Fig. 2a shows that in contrast to hypothesis H1, polycultures did not yield more biomass than monocultures (Table 2). The monoculture of *Selenastrum* (F) achieved the highest mean biomass concentration throughout the experiment (224 mg L^{-1}), outperforming all of the other monocultures and polycultures. None of the polycultures significantly outperformed the mean of their component species, their best component species, or the best overall species (all $p>0.05$). H2 was supported by a significant effect of species richness on biocrude yields (Fig. 2b, Table 2). Biocrude yield – measured by convention as the wt% of biomass – was significantly higher in the 2- (mean 30.4%) and 4-species polycultures (32.2%) than the monocultures (27.3%). *Chlorella* (B) exhibited the highest biocrude yields among the monocultures, and none of the polycultures exhibited significantly higher biocrude yields than the best species. Consistent with H3, there was a significant positive effect of species richness on the temporal stability of biomass (Fig. 2c, Table 2). The effect of species richness on stability was due to the increased stability of the four-species polyculture relative to the monocultures. However, none of the polycultures exhibited significantly higher stability than the most stable monoculture (*Chlorella*, B).

Fig. 2d shows that contrary to H4, there was no significant effect of species richness on the magnitude of biomass crashes (Table 2). The monoculture of *Selenastrum* (F) had the smallest crash magnitudes and was nearly matched by polycultures AF and ABDF. The median maximum crash magnitude was a 55% reduction in biomass in one week. The 33rd and 67th percentiles occurred at magnitudes of 45% and 60%, respectively. Contrary to H5, species richness did not significantly delay large crash events compared to either the mean of the monocultures or to the best single species (Fig. 3a). Despite finding a significant effect of species richness on crash timing (Table 2), the 2-species polycultures tended to experience crashes earlier in the experiment than the monocultures, and the ability of the 4-species polycultures to

delay crashes was marginally significant (post-hoc p=0.063). Among the monocultures, *Selenastrum* (F) was most resistant to crashes, but did exhibit one large crash beginning on week 5.

Consistent with H6, we found that the maximum proportion of invading algae decreased with species richness (Fig. 2e, Table 2). The 4-species polyculture had significantly less invading algae than the monocultures and 2-species polycultures ($p<1x10^{-4}$), but did not significantly outperform the best species (*Selenastrum*, F). Consistent with H7, we found that species richness significantly delayed invasion by unwanted species of algae (Fig. 3b, Table 2). The 2-species polycultures outperformed the monocultures at delaying invasion and the 4-species polyculture outperformed both the 2-species polycultures and the monocultures (all $p<0.001$). The 4-species polyculture significantly outperformed the best single species (*Chlorella*, B) at delaying invasion ($p<0.02$). Ponds inoculated with the 4-species polyculture remained below 1% invader algae until the last sampling, when two of the eight replicates were invaded. All of the species inoculation treatments offered some invasion resistance compared to the control ponds, which were rapidly colonized over the first week of the experiment.

Consistent with H8, we found that certain polycultures maintained more functions at higher levels of performance than any of their component species did as monocultures. Fig. 4a shows that no mono- or polyculture maximized all of the functions measured in the study. Among the monocultures, *Selenastrum* (F) had superior performance in terms of biomass production and reducing crash magnitudes, but its performance ranks for biocrude yield and invasion timing were each below the median level of performance. In contrast, the two-species polycultures AF, BD, and BF offered relatively high performance ranks for all of the functions. The four-species polyculture had high performance ranks for all functions except biomass production. Fig. 4b shows that for both the monocultures and the two-species polycultures, the mean number of functions performed above the threshold decreases steadily with increasing thresholds. The four-species polyculture maintained more functions at higher levels of performance than the monocultures did. When the performance threshold was between 40 and 80% of the maximum, the four-species polyculture performed significantly more functions above the threshold than the mean monoculture or mean two-species polyculture. Moreover, even when compared to the best monoculture at each threshold, the four-species polycultures showed superior multifunctionality (Fig. 4c). Notably, the best of the monocultures performed only four

functions above the 60th percentile threshold, but the four-species polyculture performed six out of seven functions above the 70th percentile threshold.

Discussion

Laboratory experiments have suggested that algal diversity could improve several aspects of biofuel feedstock cultivation, but this prediction has not been rigorously tested under field conditions. We experimentally tested eight hypotheses for how polycultures influence performance of outdoor biofuel feedstock cultivation (Table 1). In many cases our findings were contrary to *a priori* predictions that were based on previously published literature, and polycultures did not consistently outperform the best single species. The two well-supported hypotheses were that polycultures would decrease invasion by unwanted algae, and that polycultures would improve overall multifunctionality of the feedstocks. In the following sections we assess each of these hypotheses and discuss what our findings mean for the prospect of large-scale biofuel feedstock cultivation.

Biomass production and biocrude yield. Our experiment contradicts the prediction that algal diversity increases the production of biomass (H1). In our experiment most of the polycultures actually produced less biomass than the average monoculture (Fig. 2a) – results that are consistent with the recent laboratory experiment that used the same species pool (Narwani et al., 2016). The poor performance of polycultures in the laboratory experiment was attributable to competition among species of algae. A similar explanation is likely for the present experiment; nutrient concentrations remained high, but *Chlorella* and *Selenastrum* dramatically attenuated light, which suggests that light was a limiting resource (Fig. S5). In both the present experiment and the laboratory mesocosms, the 2-species polycultures AF and BF produced more biomass than the most diverse polyculture, but polycultures collectively underperformed relative to the best species. This finding is a contrast to the predominantly positive effects of species richness observed in biodiversity-function experiments (Cardinale et al., 2011; Hooper et al., 2005) and in several experiments performed in the context of algal biofuels (Shurin et al., 2013; Stockenreiter et al., 2011; Stockenreiter et al., 2016). This contradiction could be due the limited taxonomic diversity and functional variation used in our experiment: previous studies that reported positive effects of species richness on the production of biomass or biovolume included algae from a greater variety of taxonomic groups (e.g. diatoms, cyanobacteria, and chrysophytes) (Shurin et

al., 2013; Stockenreiter et al., 2016). Further experiments will be needed to determine whether our findings are specific to the species pool that we used or are representative of the culture conditions used in our study.

While we did not find an effect of diversity on total biomass, our experiment did support the hypothesis that species richness increases the yield of biocrude per mass of algal feedstock (H2, Fig. 2b). A similar effect has been observed in previous studies that found increased biocrude yield (Hietala et al., 2017) in polycultures compared to monocultures as well as increased lipid content (Stockenreiter et al., 2011; Stockenreiter et al., 2013), which would lead to increased biocrude yield. The positive effect of biodiversity on biocrude yield of algal feedstocks could potentially enhance overall production of biofuel from a culture, but it remains unclear whether those effects would offset or overcome a potential decrease in the total biomass production by polycultures compared to monocultures.

Stability and Crashes. Consistent with H3, biodiversity increased stability of biomass through time compared to the average monoculture in our experiment; however, polycultures did not outperform the best monoculture. Although the effect of biodiversity on stability is well documented in natural ecosystems and in experiments (Gonzalez & Loreau, 2009; Gross et al., 2014), it has only recently become a focus in the application of biodiversity to biofuels (Beyter et al., 2016; Narwani et al., 2016). Narwani and others (2016) found a similar positive effect of diversity on stability when they subjected laboratory cultures to weekly fluctuations in water temperature. The magnitude of weekly temperature change in that experiment was smaller than the daily temperature oscillations observed in the outdoor ponds of this experiment (Fig. S6). However, both experiments found that species richness tended to decrease biomass but increase the temporal stability of biomass. For instance, polyculture AF exhibited 19% less biomass and 33% higher stability than the most productive species (*Selenastrum*, F), but 53% higher biomass and 5% higher stability than the most stable species (*Chlorella*, B). This suggests that certain polycultures might offer a compromise between production and stability.

Our findings did not support the hypotheses that diversity would minimize and delay crash events relative to the monocultures (H4 & H5). Polycultures did not significantly outperform the best species (*Selenastrum*, F) in terms of minimizing or delaying crashes. Crash events are particularly important for large-scale outdoor cultivation since the culture typically needs to be re-established, which is a major expense in terms of resources and lost productivity

(National Research Council, 2012). Resistance to crash events will be a key metric for identifying mono- and polycultures that are suitable for outdoor cultivation. A previous study found that, compared to the mean monoculture, polycultures were less likely to exhibit low biomass yields over time (Narwani et al., 2016). Yet, we are unaware of any other experiments that have attempted to quantify the impact of species richness on sudden crash events in biofuel feedstock cultures. Because crash events are less likely to occur under laboratory conditions, these findings underscore the importance of testing the hypothesized benefits of biodiversity under conditions that mimic commercial production.

Lifecycle assessment (LCA) is a tool that can evaluate the impact of feedstock cultivation on the overall energy balance and resource requirements of a hypothetical algal bio-refinery. Our results suggest that describing the growth of algae in open ponds will require a more realistic approach than is typically used for LCAs. In particular, most LCAs are performed under 'steady-state' assumptions where the culture is in continual production for a large fraction of the year. Recently, some LCAs have adopted models of productivity that incorporate effects of seasonality and geography (Davis et al., 2012). While this represents an improvement over models that assume invariant productivity, our experiment shows the importance of sudden crash events and invasions for outdoor cultivation. In addition to the loss of production output, these catastrophic events will often require a cultivation pond to be drained and restarted, which increases demands on energy and resources. Our experiment shows that the risk of culture crash is not uniform through time and that this risk differs substantially among types of feedstocks. Since both small fluctuations and large crashes are inevitable consequences of cultivating algae outdoors, these aspects of temporal instability need to be explicitly represented in models describing algal cultivation.

Invasion By Algae. Our experiment supports the hypotheses that increased species richness can decrease and delay invasion by unwanted species of algae (H6 & H7). As monocultures, *Chlorella* (B) and *Selenastrum* (F) delayed invasions and decreased invader prevalence relative to the other monocultures, but both of these species were susceptible to invasions. However, most of the ponds inoculated with both *Chlorella* and *Selenastrum* remained free of invader algae for 10 weeks (BF and ABDF). The superior resistance to invasion by certain polycultures is a key finding because it suggests that biodiversity could help overcome a major challenge for cultivation at large scales.

Biodiversity can increase resistance to invasion when the resident species utilize the available resources to the extent that invader species are unable to establish and grow (Shea & Chesson, 2002). Inorganic nutrients (N and P) remained at high concentrations throughout our experiment, but both *Chlorella* and *Selenastrum* attenuated available light when they were dominant (Fig. S5). This suggests that competition for light could limit the success of invaders. Light availability is a function of the concentration of biomass in algal cultures due to self-shading (Kenny & Flynn, 2015), which means that dense cultures should be invaded more slowly than cultures with low biomass or have recently undergone a crash. This is an important finding because it underscores the importance of delaying and decreasing crashes for preventing invasion by unwanted algae.

Multifunctionality. Our experiment supports the hypothesis that polycultures can maintain more functions at higher levels of performance than monocultures (H8). Although none of the monocultures performed more than four of the seven functions above the 60^{th} percentile, the 4-species polyculture maintained six functions above the 70^{th} percentile. However, these benefits for multifunctionality occur because of strong tradeoffs among species and polycultures. For example, the four-species polyculture exhibited high performance ranks for most functions, but this benefit was offset by poor performance in biomass production. It appears that such tradeoffs are common when designing polycultures for biofuel feedstock cultivation (Godwin et al., 2017b; Hietala et al., 2017; Narwani et al., 2016; Shurin et al., 2013), so identifying polycultures that can come closer to optimizing multiple functions should be a priority for future research.

Superior multifunctionality by polycultures is an important finding because the best monocultures – *Chlorella* and *Selenastrum* – each had poor performance for at least one function. For example, *Chlorella* tended to experience crashes earlier in the experiment and was more likely to be invaded than other species combinations, and *Selenastrum* had lower biocrude yields and was invaded earlier compared to other species combinations. Thus, picking a monoculture means that at least one function will be below the median performance rank. In contrast, picking the polyculture AF, BD, or BF would result in all seven functions being performed at or above the median rank. A key consequence of these performance tradeoffs is that choosing a feedstock based on any single function (e.g. biomass production) will likely result in undesirable

493 performance in terms of other functions, but polycultures are more likely to perform all functions
494 at a high level.
495 The benefits of multifunctionality become more even important when we consider other
496 aspects of the biofuel production lifecycle that were not examined in our present study.
497 Specifically, other work has shown that biodiversity can improve nutrient-use efficiency
498 (Godwin et al., 2017b; Shurin, Mandal, & Abbott, 2014), nutrient recycling (Godwin et al.,
499 2017a), lipid content (Stockenreiter et al., 2016), and biocrude characteristics (Hietala et al.,
500 2017) in algal biofuel systems. Each of these aspects of multifunctionality is essential for algae-
501 based biofuels to be both economically feasible and sustainable. The practical importance of
502 multifunctionality is reflected in the numerous lifecycle assessments that have quantified how
503 each of these functions impacts the balance of energy and greenhouse gases over the lifecycle
504 (Frank, Elgowainy, Han, & Wang, 2013; Orfield et al., 2014; Quinn & Davis, 2015). The
505 potential for polycultures to improve multifunctionality suggests that polycultures could be
506 designed to improve performance across the biofuel lifecycle and overcome biological tradeoffs
507 exhibited by monocultures.
508 Large-scale cultivation will require feedstocks that are not only stable under outdoor
509 cultivation, but can also be harvested at a high rate through time. The rate of productivity (mass
510 per area per time) has a large influence on the feasibility of future algal biofuel systems (Quinn
511 & Davis, 2015). Although we did not harvest the algae continuously or periodically as would be
512 required to accurately estimate productivity (Kenny & Flynn, 2015), we did quantify the
513 maximum growth rates of the species compositions during the first two weeks of the experiment
514 (Supporting Information). Fig. S7 shows that the species compositions that exhibited highest
515 mean biomass also exhibited highest growth rates. Specifically, compositions B, F, and BF grew
516 more quickly (0.52 - 0.54 d^{-1}) than did compositions A and AD (0.30 - 0.34 d^{-1}). These estimates
517 of maximum growth rates suggest that *Selenastrum*, *Chlorella*, and their bi-culture (BF) could
518 achieve high productivity under outdoor conditions. Further experiments will be required to
519 determine which species compositions and harvesting regimes lead to the highest and most stable
520 productivity under realistic conditions.
521 Despite the growing body of literature highlighting the potential for biodiversity to
522 improve algal biofuel production, our study is one of a small number that have experimentally
523 tested these predictions in field conditions. Although the studies performed to date were by no

means exhaustive, the collective evidence from these experiments suggests that the effects of biodiversity on biomass production are likely smaller than what has been forecast. At the same time, there is a growing body of evidence suggesting that biodiversity has other benefits besides biomass production, including temporal stability, resistance to unwanted pest species, more efficient use of nutrients, and greater levels of multifunctionality. The practical importance of biodiversity and multifunctionality will depend upon how these functions impact the long-term balance of energy and cost in commercial-scale cultivation. Determining the net impact of biodiversity will require (1) additional experiments that directly test the hypothesized benefits of biodiversity under relevant conditions and (2) more realistic LCAs that use empirical data from these experiments to evaluate the performance of different feedstocks in terms of energy return on energy invested and other metrics of environmental sustainability.

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Conflict of Interest

There are no conflicts of interest.

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676

Data Statement. The entire dataset is available in the Supporting Information.

Tables

Table 1. List of hypotheses regarding the potential for biodiversity to improve algal feedstock cultivation. Each prediction is evaluated in terms of (1) the average at each level of species richness, (2) the capacity for at least one polyculture to outperform its best component species and (3) the to capacity for at least one polyculture to outperform the best single species examined in the experiment. 'X' Means that the prediction was not supported. ✓Means that the prediction was supported and was statistically significant ($p<0.05$).

Hypotheses. Compared to monocultures, certain polycultures:	Compared to…		
	…the mean of their component species	…their best component species	…the best species in the experiment
H1: Increase biomass	X	X	X
H2: Increase biocrude yield	✓	X	X
H3: Increase stability	✓	X	X
H4: Decrease crash magnitude	X	X	X
H5: Delay crashes	X	X	X
H6: Decrease invasive algae	✓	X	X
H7: Delay invasive algae	✓	✓	✓
H8: Maintain more functions at	✓	✓	✓

Table 2. Results of general linear models and logistic regression models used to test hypotheses H1 through H7. Effects of species combination (Combo) were nested in species richness (SR). Subscripts are used to display the numerator and denominator degrees of freedom (e.g. $F_{i,j}$). [b] Denotes spatial block was included as a random effect in the minimum adequate model and [p] denotes pond identity was included as a repeated measures random effect in the minimum adequate model.

Hypotheses	Response variable	SR	SR|Combo	Time	SR*Time	Combo*Time
H1	Biomass [p]	$F_{2,59}$=1.91, p=0.16	$F_{8,59}$=2.77, p<0.02	$F_{1,61}$=6.20, p<0.02	$F_{2,61}$=0.17, p=0.85	$F_{8,61}$=3.37, p<0.01
H2	Biocrude yield [b,p]	$F_{2,80}$=7.29, p<0.02	$F_{8,82}$=3.58, p<0.02	$F_{1,83}$=18.62, p<0.001	$F_{2,77}$=0.70, p=0.50	$F_{8,73}$=0.65, p=0.74
H3	Stability	$F_{2,61}$=5.45, p<0.01	$F_{8,61}$=4.66, p<0.001	NA	NA	NA
H4	Maximum crashes [b]	$F_{2,61}$=1.34, p=0.27	$F_{8,61}$=4.14, p<0.001	NA	NA	NA
H5	Crash timing	p<0.02	$p<1x10^{-9}$	$p<1x10^{-15}$	p=0.29	p=0.56
H6	Maximum invader algae [b]	$F_{2,58}$=12.20, $p<1x10^{-4}$	$F_{8,58}$=10.88, $p<1x10^{-8}$	NA	NA	NA
H7	Invasion timing (>1%)	$p<1x10^{-15}$	$p<1x10^{-15}$	$p<1x10^{-15}$	p=0.8	NA

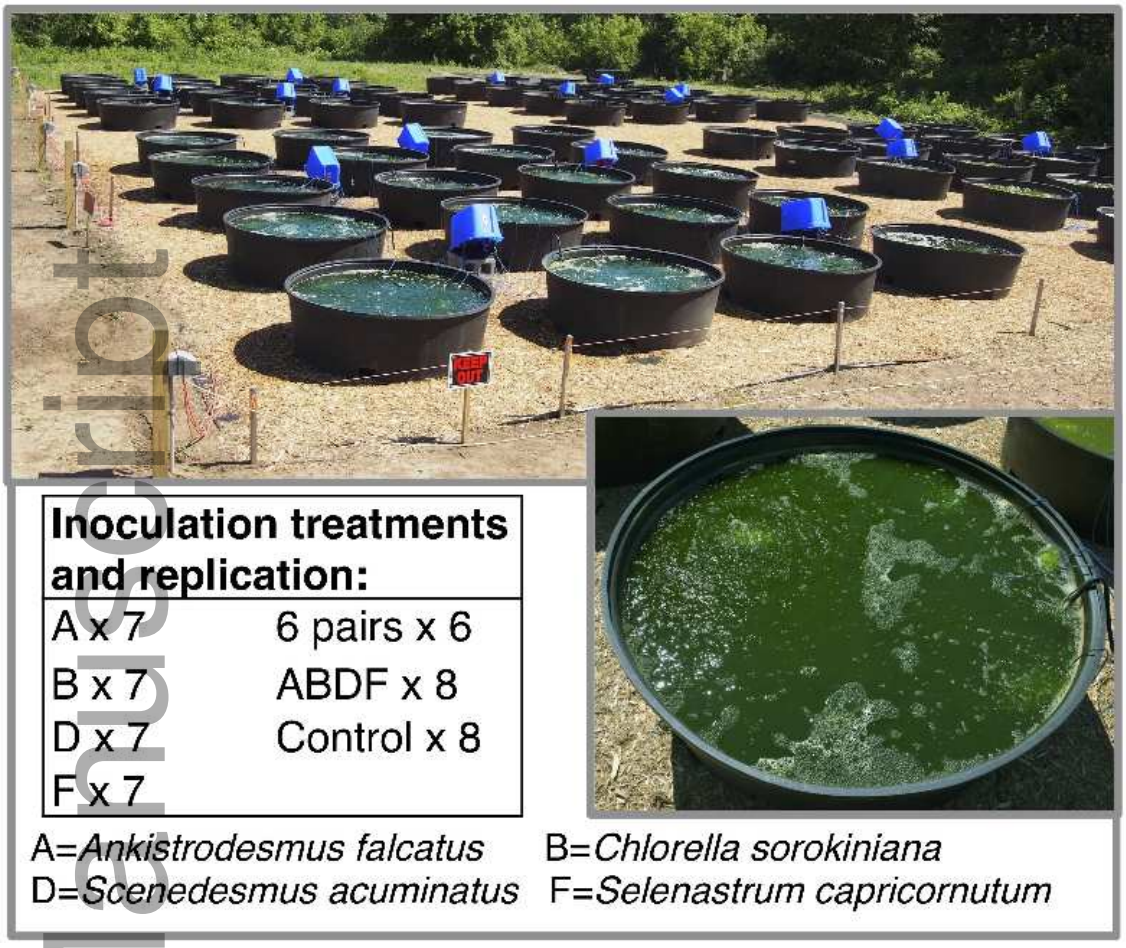
Figure Captions

Fig. 1. Photographs of the experimental 1,100L outdoor ponds located at the Edwin S. George Reserve near Pinckney, MI, USA. Inset text summarizes the experimental design and replication.

Fig. 2. Box-whisker charts showing the mean biomass (a), biocrude yield (b), biomass stability (c), maximum biomass crash magnitude (d), and maximum proportion of invader algae (e) for replicate ponds in each inoculation treatment. Blue styling denotes monocultures, orange denotes 2-species polycultures, green denotes the 4-species polyculture, and grey denotes the control treatment (no inoculum). The horizontal lines in each box represent the median, the edges of the box represent the 25^{th} and 75^{th} percentiles, the whiskers represent the maximum and minimum values, and filled circles represent values more than twice the interquartile distance from the median. In panels a-c the dashed horizontal lines show the mean for each level of species richness. In panel d the dashed line represents the overall median. Species codes are listed in Fig. 1.

Fig. 3. Percent of replicate ponds that crashed on or before each date (a). The threshold for crashes was a 55% reduction in biomass in 7 days, which was the median for the maximum crash size observed in each pond. Occurrence of invader algae in the ponds through time (b). Error bars represent one standard error of the mean for replicate ponds (n=6 to 8 each).

Fig. 4. Heatmap showing performance tradeoffs among the monocultures and polycultures (a). All performance ranks are ordered to that warmer colors represent more desirable performance. Plots of the number of functions performed above each threshold, with separate styling for the mean at each level of species richness (b) and the best performer at each level of species richness (c). Error bars in panel b denote the standard error. In panel c, lines were jittered vertically to improve readability: monocultures were moved up slightly and the 4-species culture was moved down slightly with respect to the 2-species polycultures. In panels b and c, * symbols indicate significantly higher performance of the 4-species polyculture compared to the monocultures ($p<0.05$), as determined by randomization tests (see Methods, Fig. S4).



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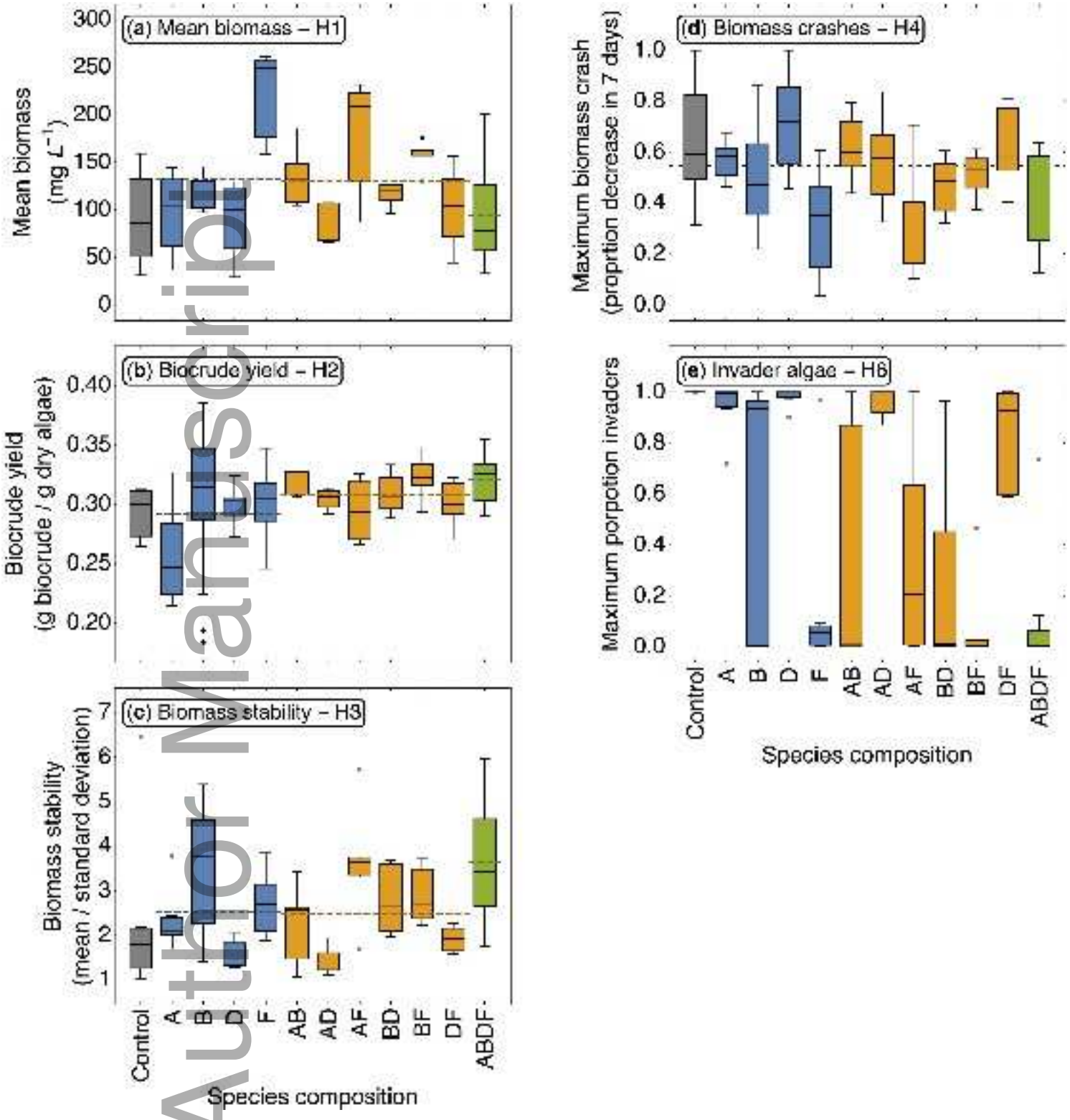

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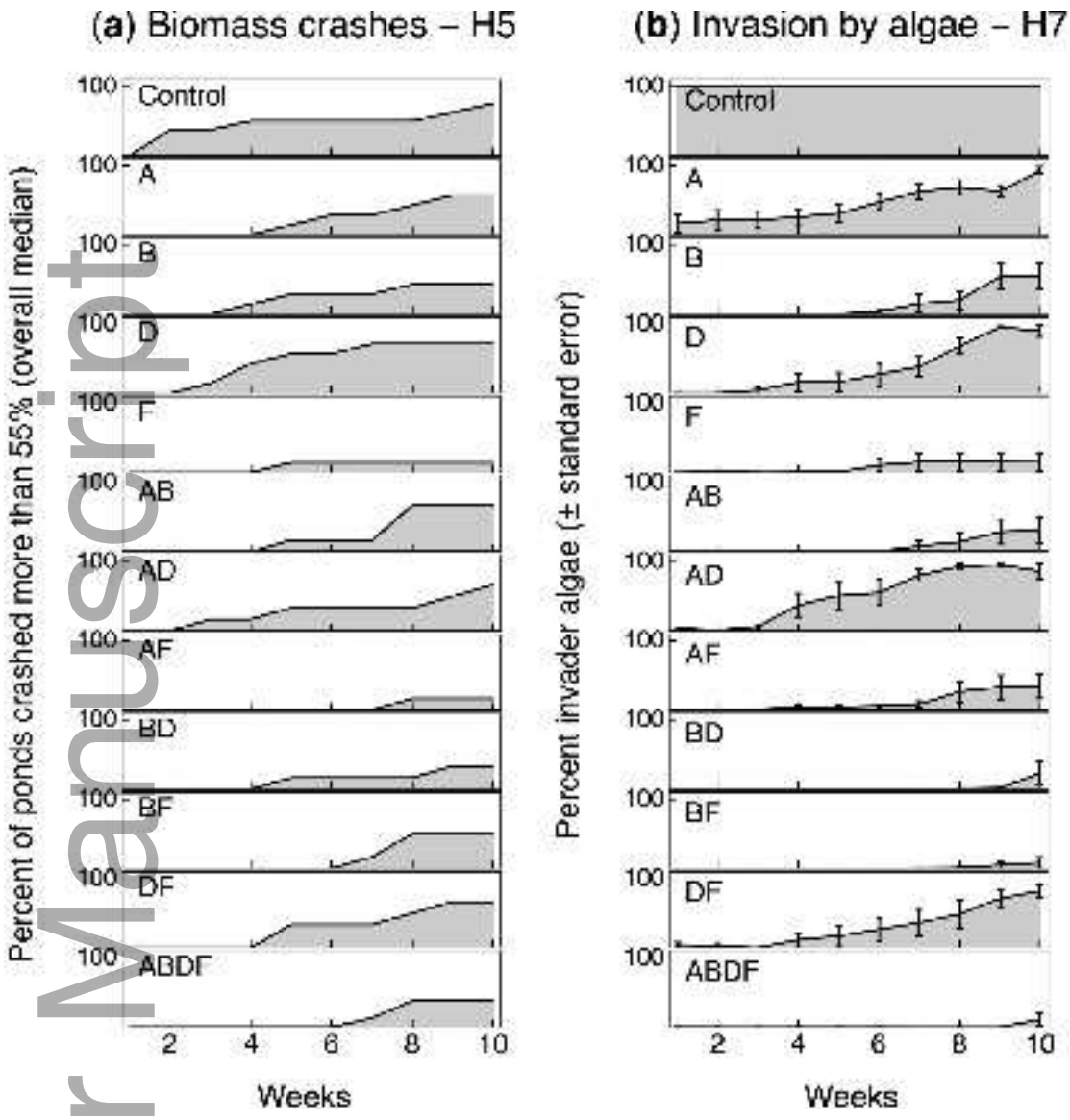

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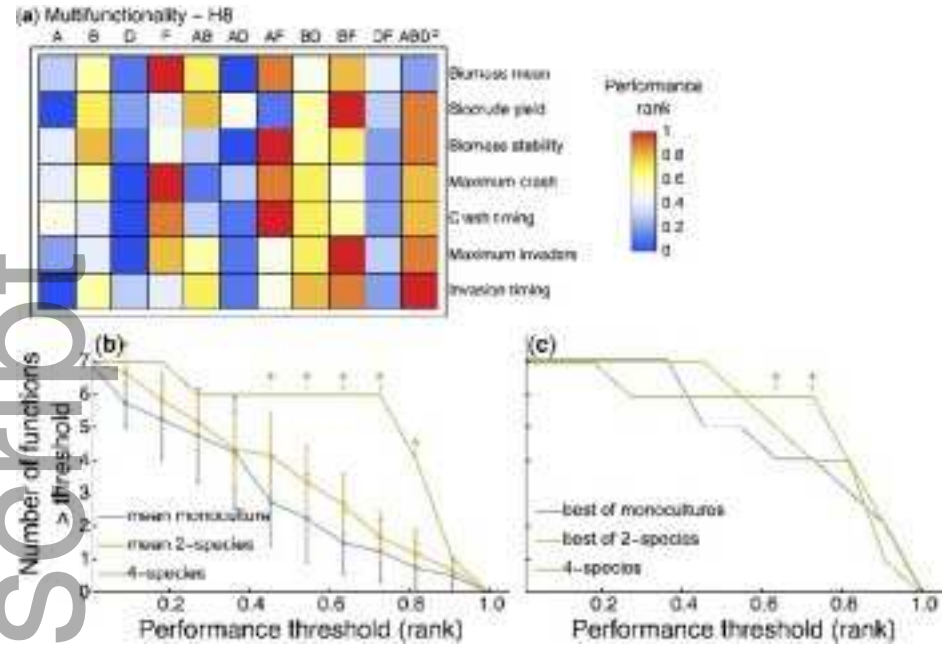

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