Title: Algal polycultures enhance coproduct recycling from hydrothermal liquefaction

Authors: Casey M. Godwin ^{a*}, David C. Hietala ^b, Aubrey R. Lashaway ^a, Anita Narwani ^{a, 1}, Phillip E. Savage ^{b, c}, and Bradley J. Cardinale ^a

Affiliations:

^aSchool of Natural Resources And Environment, University of Michigan, 440 Church Street, Ann Arbor, MI 48109, USA

^bDepartment of Chemical Engineering, University of Michigan, 2300 Hayward Street, Ann Arbor, MI 48109, USA.

^cDepartment of Chemical Engineering, Pennsylvania State University, 158 Fenske Laboratory, University Park, PA 16802, USA.

¹ Present address: Eawag, Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland

* Corresponding Author at School of Natural Resources And Environment, University of Michigan, 440 Church Street, Ann Arbor, MI 48109, USA, cgodwin@umich.edu

Abstract

The aim of this study was to determine if polycultures of algae could enhance tolerance to aqueous-phase coproduct (ACP) from hydrothermal liquefaction (HTL) of algal biomass to produce biocrude. The growth of algal monocultures and polycultures was characterized across a range ACP concentrations and sources. All of the monocultures were either killed or inhibited by 2% ACP, but polycultures of the same species were viable at up to 10%. The addition of ACP increased the growth rate (up to 25%) and biomass production (53%) of polycultures, several of which were more productive in ACP than any monoculture was in the presence or absence of ACP. These results suggest that a cultivation process that applies biodiversity to nutrient recycling could produce more algae with less fertilizer consumption.

Keywords: Algal biofuels; biodiversity; nutrient recycling; hydrothermal liquefaction; coproduct recycling

1. Introduction

Algal biofuels offer the promise of simultaneously reducing both fossil fuel consumption and the net production of greenhouse gases. Although improvements in production and processing technologies have begun to move algal biofuels towards systems that are more economically feasible in terms of their energy return, the high demand for fertilizer inputs remains a major obstacle to large-scale commercial production (2010; Clarens et al., 2010; Pate et al., 2011; Peccia et al., 2013). Recent estimates (Pate et al., 2011) suggest that replacing diesel transportation fuel with algal biofuel from direct lipid extraction would increase U.S. consumption of nitrogen fertilizer by more than 400% and phosphorus fertilizer by more than

200% unless those nutrients are recycled within the biofuel lifecycle. It is paramount that algal biorefineries efficiently retain and recycle nitrogen and phosphorus to avoid exacerbating nutrient-driven eutrophication (Bennett et al., 2001) and disruption of global biogeochemical cycles (Gruber & Galloway, 2008). Nutrient recycling is not only essential for minimizing these environmental impacts, but would also improve commercial feasibility of algal biofuels by reducing the demand for fertilizer (US DOE 2010; National Research Council, 2012).

Several technologies have been developed to convert algal biomass into biocrude oil or other precursors of biofuels (National Research Council, 2012). One such technology called hydrothermal liquefaction (HTL) converts whole, wet algal biomass into biocrude, producing more oil per mass of algae than direct lipid extraction methods, and avoiding energy costs associated with drying the biomass prior to conversion (Biller & Ross, 2011; Valdez et al., 2012). The process for sustainable algal biocrude production using HTL can be conceptualized as a cycle of biomass cultivation, biocrude production, and nutrient recovery from the HTL processing (Figure A1). Algal biomass production occurs in open raceways or closed photobioreactors, both of which have high demands for fertilizer inputs (principally inorganic nitrogen and phosphorus). Upon harvest, the culture is dewatered to high solids content (5-15% by mass) and processed by HTL. Because the reaction occurs in aqueous medium, HTL also generates a nutrient-rich aqueous-phase coproduct (ACP) that contains most of the N and P from the algal biomass (Bagnoud-Velasquez et al., 2015; Costanzo et al., 2015; Garcia Alba et al., 2013a; Valdez et al., 2012) as well as micronutrients. The ACP can potentially be recycled into the depleted medium to grow more algae (Biller et al., 2012; Jena et al., 2011; Nelson et al., 2013; Orfield et al., 2014) or heterotrophic bacteria. Although efficient coproduct recycling would dramatically reduce fertilizer consumption, previous studies have suggested that ACP may

be inhibitory to algae at concentrations of 1-2% and would require substantial dilution before it could be recycled (Biller et al., 2012; Jena et al., 2011), leading to diversion of nutrients from the process (purging). In practice, the dilution of ACP is constrained by the concentration of biomass at the time of harvest and the conditions used during HTL (Hietala et al., 2016). For example, cultures with 0.02 and 0.1% dry mass content (0.2 and 1 g L^{-1})(National Research Council, 2012), processed by HTL with 5% mass loading, yield equally concentrated coproduct volumes that are approximately 0.4 and 2% of the original culture volume, respectively. Thus, identifying algal strains that are tolerant of ACP within this range of concentrations is critical because the efficiency of the recycling pathway will be optimized only by maximizing the fraction of ACP that can be added to the depleted medium at each round of the cycle.

Since the 1970s, most research on algal biofuel production has been focused on identifying species with the greatest potential for fuel production (Griffiths & Harrison, 2009; Mata et al., 2010) or genetically modifying strains to enhance desirable properties (Georgianna & Mayfield, 2012; Rosenberg et al., 2008). Although this work has identified species or strains with high yields of biomass or lipids, the development of large-scale algal biofuels has been challenged by cultures that get ruined by disease, environmental perturbations, and invasion by non-focal species (National Research Council, 2012; Carney & Lane, 2014). Given these challenges to large scale cultivation, several recent studies have suggested that multi-species cultures (polycultures) of algae might be useful for improving algal biofuel systems if communities could be designed to meet particular needs (Newby et al., 2016; Shurin et al., 2013; Smith et al., 2015). More than two decades of ecological research has shown that, compared to monocultures of a single species, multi-species polycultures of plants and algae can produce more biomass (Cardinale et al., 2006), use resources more completely (Cardinale, 2011; Naeem

et al., 1994), and better resist invasive species and pathogens (Mitchell et al., 2002). Although polycultures could improve other aspects of algal biofuel production, it is unclear whether polycultures of multiple species could overcome the inhibitory effects of ACP recycling (Levine et al., 2013; Pham et al., 2013). Resource partitioning and competitive interactions might improve nutrient use efficiency by polycultures (Cardinale, 2011; Cardinale et al., 2007), but the impact of these mechanisms would be limited if the species are inhibited or killed by ACP. However, if species are more tolerant of ACP when grown as polycultures due to synergies like facilitative interactions, then diverse polycultures could potentially outperform their component species in coproduct recycling.

The purpose of this experiment was to characterize the ability of algal monocultures and polycultures to grow using coproduct recycled from HTL of algal biomass. The experiment focused solely on the recycling pathway and ACP tolerance because this pathway is essential to the sustainability and economic feasibility of algal biofuels; yet it has been studied far less than production of biomass or lipids. This study simulated a recycling pathway by using ACP taken after HTL processing of algal species grown alone as monocultures and together as polycultures, and adding the ACP to the growth medium to grow additional algae as monocultures and polycultures. The objectives of this study were to 1) determine if polycultures were more tolerant of ACP than monocultures of their component species 2) test whether polycultures could produce as much biomass as the most productive monocultures when recycling ACP and 3) determine whether certain species or combinations of algae produce more or less inhibitory ACP.

2. Material and Methods

2.1 Species Selection and ACP Production

The experiment described here (hereafter called the ‘ACP recycling experiment’) is a complement to an earlier experiment completed by Narwani et al. (2016) (the ‘mesocosm experiment’) that was designed to test whether algal polycultures improve the productivity, stability, and quality of biocrude relative to monocultures grown in the same fresh medium (Figure 1). The mesocosm experiment focused on six species of freshwater green algae that (a) were identified as high value for lipid production by the U.S. Department of Energy's Aquatic Species Program (US DOE, 1998) and included in the Solar Energy Research Institute's microalgae collection, and (b) have shown evidence of overyielding of biomass production in polycultures (Fritschie et al., 2014). In addition, because the inadvertent release of genetically modified strains or non-native species from commercial production could disrupt local ecosystems, focal species for the mesocosm experiment were also chosen to represent naturally occurring, dominant species of algae found in lakes across the continental U.S. (US EPA, 2012). The selected species were: *Ankistrodesmus falcatus* (abbreviated as A), *Chlorella sorokiniana* (B), *Pediastrum duplex* (C), *Scenedesmus acuminatus* (D), *Scenedesmus ecornis* (E), and *Selenastrum capricornutum* (F). Full details of the mesocosm experiment are given in Narwani et al. (2016). Briefly, the algae were cultured in 9.5 L mesocosms that were diluted at 30% week⁻¹ with a widely used growth medium (Bold-3N (Bold, 1949)) and illuminated with fluorescent lamps. The mesocosm experiment was performed in two temporal blocks (Figure 1), each with a factorial design of species combination and water temperature regimes (constant at 22°C or variable from 17-27°C). The mesocosms were inoculated as monocultures of each species or as polycultures of two, four, or six species using a substitutive design where the total cell density of the inoculum was constant across all treatments. Once the mesocosms reached steady-state biomass, algae were harvested every 7 days for 7-8 weeks and biomass was

concentrated by settling, centrifugation, and drying. HTL was performed at 350 °C for 20 minutes using a 5% dry mass loading (Narwani et al., 2016). Following HTL, the ACP was separated and frozen at -20°C until use in the recycling experiment presented here.

2.2 ACP Recycling Experiment Design

In the ACP recycling experiment described in this paper, ACP that resulted from HTL of biomass from monocultures and polycultures in the mesocosm experiment (hereafter ACP source cultures) was used to grow additional algae as monocultures and polycultures (recycler cultures, Figure 1). The ACP recycling experiment used all six species as monocultures, the five most productive two-species polycultures from the mesocosm experiment, the five most productive four-species polycultures, and the six-species polyculture. The ACP recycling experiment was executed in two temporal blocks, corresponding to the two temporal blocks from the mesocosm experiment. Within each temporal block, the design had two experimental blocks that corresponded to the two temperature treatments from the original mesocosm experiment (constant or variable). Due to low biomass production by some monocultures in the mesocosm experiment (species C and E), the ACP used for each source culture within a single experimental block of the ACP recycling experiment was pooled evenly from all replicate mesocosms and weeks in the mesocosms experiment.

2.3 ACP Recycling Cultures

Algae were cultured in Bold-3N medium containing 8.82 mmol-N L⁻¹ as nitrate and 1.72 mmol-P L⁻¹ as phosphate. The ACP was added to nutrient-replete medium (instead of depleted medium or nutrient-free water) to isolate the inhibitory effects of ACP from its inorganic nutrient content. ACP treatments consisted of a control without ACP added, and seven concentrations of ACP additions ranging between 0.1% and 2.0% by volume (1000x to 50x

effective dilution in Bold-3N medium). This range of ACP concentrations was chosen because 1) this is the range of dilutions that would result from recycling the entire volume of ACP into the culture medium, provided the cultures contain 50 to 1000 mg L⁻¹ biomass and HTL is performed with 5% mass loading (Hietala et al., 2016; Narwani et al., 2016), and 2) previously published studies have shown apparent toxicity in the range of 0.2 to 1% ACP (Barreiro et al., 2015; Biller et al., 2012).

Three replicate 1 mL cultures were inoculated at each dilution with an initial concentration of 500 cells mL⁻¹ in a substitutive design such that the initial cell density was constant across all treatments (i.e. each species in a 2-species polyculture was inoculated at 250 cells mL⁻¹). The substitutive design based on total cell density ensures that all population sizes are equal within a polyculture and that the effect of species richness on culture viability is not confounded in the initial total population size. In total, each combination of ACP source, recycler, and dilution was replicated in 12 cultures (3 technical replicate wells in two temporal blocks and two experimental blocks). The culture plates were sealed with sterile gas-permeable membranes (Breathe-Easy, Excel Scientific) and incubated on orbital shaker tables (120 rpm) at a constant temperature of 20°C beneath fluorescent light fixtures that delivered at least 100 $\mu\text{mole m}^{-2} \text{s}^{-1}$ at the surface of the plates (16h light, 8h dark).

2.4 Fluorescence Monitoring and Cell Counts

Chlorophyll-a fluorescence was used as a proxy for algal biomass. Fluorescence is a suitable proxy for this experiment because it can be measured non-destructively in a large number of experimental units and the ratio of biomass to fluorescence does not differ significantly among the focal species (ANOVA $F_{5,42}=2.03$, $p=0.094$). Chlorophyll-a fluorescence was monitored in each well using a BioTek Synergy H1 plate reader with an excitation

wavelength of 435 nm and an emission wavelength of 685 nm. The incubations were terminated after 20 days in temporal block 1 and 18 days in block 2 because evaporative losses began to exceed 20% of the culture volume. By the end of the incubations, the median growth rate of the cultures was less than 0.0024 h^{-1} for block 1 and less than 0.0031 h^{-1} for block 2, which suggests that algal population growth was nearly stationary. Immediately following the final fluorescence measurements, cultures were fixed with 1% buffered formalin. To determine which species dominated the polycultures at the end of the experiment, species-specific biovolume estimates (Narwani et al., 2016) were used to describe changes in species composition in response to ACP addition. The biovolume of each species was quantified in treatments where the recycler culture received ACP from the same species combination. At least 500 cells were enumerated from each of two randomly selected wells at 0, 0.5, 1, and 2% ACP.

2.5 Analyses

The maximum fluorescence measurement for each culture well was used to quantify the cumulative production of biomass during the ACP recycling experiment. To approximate the maximum growth rate of each well without assuming a model of population growth, the exponential growth rate was calculated over each set of three consecutive fluorescence measurements and the maximum observed growth rate was used for the analyses. Recycler cultures were classified as killed if their maximum fluorescence was less than twice the highest fluorescence observed among 264 sterile blank wells (equal to 72 relative fluorescence units, RFU). To normalize the responses of different species to ACP, log response ratios were computed as the maximum fluorescence (LRR_F) or maximum growth rate (LRR_{GR}) for each well divided by the mean from the three control wells (with algae, but no ACP added) on the same culture plate. Negative response ratios indicate growth inhibition in the presence of ACP, ratios

near zero indicate tolerance (but no enhancement of growth), and positive ratios indicate enhanced growth in the presence of ACP. To test whether polycultures actually produced more biomass or grew more quickly than monocultures, the maximum fluorescence and growth rates were analyzed using a linear mixed model with recycler culture and ACP source culture as fixed effects and random effects of the experimental blocks nested in the temporal blocks. The linear mixed model was performed using the R package 'lme4'. To evaluate whether the maximum fluorescence or growth rate of each polyculture was significantly greater than that of its component species or greater than all six species, post-hoc tests were performed at each dilution level of ACP ($\alpha=0.05$).

3. Results and Discussion

3.1 Tolerance and Response to ACP

Algal polycultures were more tolerant of high ACP concentrations than monocultures, and polycultures exhibited higher fluorescence and growth rates in 2% ACP than in fresh medium without ACP. This outcome is apparent in the heatmap of Figure 2, which shows qualitatively that most of the monocultures were inhibited or killed by high concentrations of ACP (negative log response ratios indicated by red), whereas ACP typically enhanced the growth of polycultures (positive response ratios indicated by green). Of the monocultures, only *Chlorella* (B) exhibited tolerance or a positive response to any ACP source at 2%, but it was inhibited by ACP recycled from its own biomass. In contrast, at 2% ACP, the four- and six-species recycler cultures exhibited mostly positive response ratios for maximum fluorescence (LRR_F) and maximum growth rate (LRR_{GR}) across all ACP sources (Figure 2). When recycling their own ACP at 2%, *Chlorella* (B) monocultures showed decreased maximum fluorescence (-35%) and reduced growth rate (-9%) relative to the control, but the six-species polyculture

showed increased maximum fluorescence (61%) and growth rate (20%) relative to the control. At lower ACP concentrations, the positive effect of species richness on LRR_F and LRR_{GR} was smaller and the polycultures had similar responses to their best species (Figure A2). Across all concentrations of ACP, the effect of ACP source culture on LRR_F and LRR_{GR} was inconsistent among recycler cultures and was small compared to the differences among recycler cultures.

Contrary to some prior observations that ACP toxicity can limit the potential for nutrient recycling with monocultures (Biller et al., 2012; Jena et al., 2011), this experiment shows that diverse cultures respond positively to ACP recycling. The prior finding that direct recycling by algae is not feasible at concentrations of 1-2% without pre-treatment or purge (Jena et al., 2011; Nelson et al., 2013; Zhang et al., 2014) is supported by only a few studies that have experimentally examined the response of multiple species of algae (grown as monocultures) to ACP (Barreiro et al., 2015; Biller et al., 2012). The results for monocultures are consistent with recent studies showing that ACP does not inhibit all species to the same degree (Barreiro et al., 2015; Garcia Alba et al., 2013a) and that the inhibitory effects can be temporally transient (Garcia Alba et al., 2013b; Zhang et al., 2016). The results presented here also show that the benefits provided by species richness for ACP recycling actually increase with the concentration of ACP (up to 10%). The key implication of this finding is that diverse polycultures would require less dilution of ACP at each cycle than monocultures, thereby minimizing the diversion of nutrients from the lifecycle and the need for supplemental fertilizer inputs.

3.2 Effect of ACP on Growth Rate and Biomass Production

Not only did polycultures show greater tolerance to ACP than monocultures (Figure 2), polycultures also had higher maximum fluorescence and higher maximum growth rates compared to monocultures at high ACP concentrations. Figure 3 shows that, when recycling 2%

ACP from all sources, 10 of 11 polycultures had higher adjusted mean fluorescence (6 were significantly higher) than their component species (overyielding) and 5 polycultures had higher fluorescence (2 significantly) than all of the monoculturespecies (a phenomenon called ‘transgressive overyielding’). Among the monocultures, only *Chlorella* (B) hadmean maximum fluorescence at 2% ACP that was comparable to the 0% ACP control. Of the polycultures that exhibited transgressive overyielding at 2% ACP, the mean adjusted fluorescence was 9,862 RFU, compared to 8,507 for *Chlorella* (16% increase). The highest adjusted mean fluorescence for a monoculture at any ACP concentration was 9,753 RFU (*Selenastrum* (F) at 1% ACP). The number of polycultures that exhibited overyielding in maximum fluorescence declined at lower ACP concentrations and only two polycultures exhibited overyielding in the absence of ACP (though not significantly). When examining only self-recycling treatments (species or species combinations recycling their own ACP), 8 polycultures exhibited transgressive overyielding at each level of ACP between 0.33 and 2% (Figure 3). There was a similar effect of species richness on maximum growth rates, with 10 polycultures exhibiting overyielding (9 significantly) and 6 exhibiting transgressive overyielding (5 significantly) at 2% ACP from all sources (Figure A3). Of the polycultures that exhibited transgressive overyielding for growth rate at 2% ACP, the adjusted mean growth rate was 18% higher than that of *Chlorella*(B) (0.058 h^{-1} versus 0.049 h^{-1}). Although the four-species polycultures typically had higher fluorescence than the two-species polycultures, there was not a consistent advantage of the six-species polyculture versus those with four species.

Overyielding by polycultures when recycling ACP is a critical finding because superior tolerance and positive log response ratios alone do not necessarily guarantee a meaningful advantage in terms of biomass production rate or yield. Polycultures seldom exhibited

overyielding of biomass in the absence of ACP, both in the present study and the original mesocosm experiment (Narwani et al., 2016). A supplemental experiment confirmed that this overyielding at high ACP concentration was not the result of fertilization by the N and P found in ACP (Appendix A, Table A1). Since all of the monocultures were inhibited by the majority of the ACP sources when grown alone (negative LRR in Figure 2), it is surprising that diverse polycultures were more productive using 2% ACP than the best species was in the absence of ACP. This is particularly surprising since the recycler polycultures were dominated by a single species at 2% ACP (typically *Chlorella* (B), Figure A5), but previous studies have shown that polycultures can exhibit overyielding even when a single species is dominant and the species responsible for facilitation is rare (Fritschie et al., 2014).

High ACP concentrations induced a time lag for several days, after which the cultures had high growth rates and their biomass surpassed that of the control cultures. This effect of ACP on population growth has been reported previously (Garcia Alba et al., 2013b) and could be due to a reduction in initial population size caused by ACP, adaptation by algae to become more tolerant, or a decrease in the concentration of inhibitory factors with time. Although the cause of the lag period is uncertain, the subsequent high growth rates and high viability indicate that diverse polycultures overcome the initial inhibitory effects of ACP when present.

It is presently unclear why the addition of ACP led to overyielding, or why polycultures had higher viability than any of their component species. Resource partitioning and interspecific facilitation are the most common explanations for overyielding. Facilitation could explain why species that were each inhibited or killed by 2% ACP as monocultures could still tolerate the same conditions as a polyculture. One possible mechanism of facilitation is that mixotrophic algae (Barreiro et al., 2015; Levine et al., 2013; Zhang et al., 2016) or heterotrophic microbes

associated with different species of algae could have consumed or degraded dissolved organic compounds that inhibited growth (Faeth et al., 2016). Investigating the mechanisms underlying these outcomes is an important direction for further research because, if these effects of biodiversity translate to larger scales of cultivation, they represent an attractive ‘win-win’ scenario for sustainability and fuel production.

3.3 Effect of ACP on Culture Viability

All of the monocultures had reduced viability at high concentrations of recycled coproduct, but viability increased with species richness when they were grown in polycultures. This effect of species richness can be seen across ACP sources and also when the cultures are recycling only their own ACP (Figure 4). Across all ACP sources, each of the four- and six-species recycler cultures had significantly higher viability at 2% ACP than any single species (G-test with Williams’ correction $\chi^2 = 11.1$, $p < 0.02$). Three of the four-species polycultures and the six-species polyculture had 100% viability at 2% ACP (out of 84 to 138 replicate cultures for each polyculture). Because some recycler cultures were highly tolerant of 2% ACP, an additional experiment was performed at higher ACP concentrations using a limited number of recycler cultures (Appendix A, high-concentration ACP recycling trials, Figure A4). Across the range of ACP concentrations used in the high-concentration ACP recycling trials, the two most tolerant monocultures (*Chlorella* (B) and *Selenastrum* (F)) had LD₅₀ (concentration that killed 50% of the replicates) of 4.0% and 4.5% ACP, respectively. The polyculture consisting of A-C-D-F had higher viability than the monocultures (LD₅₀ of 5.4% ACP) and the polyculture A-B-C-D had LD₅₀ of 10.1% ACP.

Diverse polycultures were also less likely to be killed by ACP recycling than monocultures, which implies the possibility that commercial cultures might be less susceptible to

catastrophic population crashes as a result of recycling. It is important to note that due to the substitutive design used for the biodiversity treatment, the population size of each species was smaller in polyculture than in monoculture. Therefore, the increased viability of polycultures is not explained by higher initial cell density. The ability of select polycultures to tolerate ACP concentrations above the approximate maximum recycle scenario of 2% would be particularly important if the concentrations of inhibitory compounds accumulate after multiple rounds of recycling. Presently, it is not known how the effects (both positive and negative) of ACP on biomass production accumulate over repeated cycles of biomass cultivation, HTL, and recycling. To date, only two studies have evaluated the effect of repeated recycling. One study found that, following a lag period, biomass production by *Desmodemus* sp. was not diminished after five cycles of ACP addition (Garcia Alba et al., 2013b). The other study found that recycling the aqueous-phase from hydrothermal carbonization enhanced biomass production in the first cycle, but biomass was diminished in the second cycle (Levine et al., 2013). It will be critical to determine if the effect of the cumulative ACP concentration is weaker or stronger than the effect of a single addition of ACP at the same concentration (e.g. three cycles with 1% ACP addition versus a single addition of 3% ACP). If the effect of repeated addition of ACP is less than a single addition of the equivalent concentration, the results presented here suggest that the loss of nutrients during fuel extraction, as opposed to toxicity (Faeth et al., 2016), could potentially be the most important determinant of lifecycle nutrient efficiency.

3.4 Selecting Polycultures For Recycling Pathways

All of the polycultures with at least 4 species were well suited for ACP recycling. Even though the source cultures differed substantially in their biomass production (Narwani et al., 2016) and ACP nutrient content (Table A1), there were no consistent effects of ACP source

identity on all recycler cultures (Figure 2). This result means that both self-recycling (e.g. ABCD recycling coproduct from ABCD) and more complex designs (e.g. ABCD recycling coproduct from F) are feasible. However, because some of the diverse polycultures were more productive using 2% ACP than any of the monocultures, a self-recycling design with a polyculture would likely be most practical. Initially, a monoculture of *Selenastrum* (F) or *Chlorella* (B) could be beneficial for production using fresh nutrients, but after the first cycle of ACP recovery, switching to a polyculture could increase production and decrease the likelihood of crop loss. Furthermore, exposure to ACP had strong effects on species composition, and effectively selected for dominance by the species that were most productive in the absence of ACP (*Chlorella* (B) and, to a lesser extent, *Scenedesmus acuminatus* (D) and *Selenastrum* (F), Figure A5). Over repeated cycles of biomass production, HTL, and recycling, the selective effects of ACP would likely reinforce the species composition according to this hierarchy of tolerance. Repeated inoculation of the rare taxa may be needed to prevent stochastic extinctions and maintain the benefits of the polyculture. Therefore, a self-recycling design using a diverse polyculture that includes *Chlorella* (B) would be most favorable in terms of both biomass production and nutrient recycling.

This experiment simulated recycling by diluting ACP into nutrient replete medium with the intention of isolating the inhibitory effects of ACP from its nutrient content. Accordingly, the results from this experiment are not necessarily comparable to other studies that were performed by diluting ACP into medium with reduced nutrient content (Barreiro et al., 2015; Garcia Alba et al., 2013a; Jena et al., 2011), or in nutrient-free water (Biller et al., 2012; Zhang et al., 2016). When ACP is recycled into depleted medium or water and the nutrient content is less than the original medium, algal production is often suppressed, but it is not possible to separate the

negative effects of nutrient limitation from those of toxicity. Studies have demonstrated that, when ACP is diluted into partially depleted medium such that the final concentration of nutrients (N and P) is unchanged from the original medium, the effect of ACP on algal production is neutral (Garcia Alba et al., 2013a; Garcia Alba et al., 2013b) or even positive (Barreiro et al., 2015; Selvaratnam et al., 2015). Although recycling ACP into depleted medium is arguably the most realistic model for nutrient recycling in commercial biofuel production, this methodological difference does not diminish the key finding that monocultures responded negatively and polycultures responded positively under identical ACP treatments. Because inorganic nutrients were supplied at high concentrations in all treatments, the findings presented here suggest that the responses of monocultures and polycultures to coproduct are likely attributable to either its toxic components (negative effects) or organic compounds (Faeth et al., 2016) that facilitated mixotrophy (positive effects). This finding underscores the need for mechanistic experiments to identify the components of ACP that are inhibitory or beneficial to the growth of algae, and to characterize the biological mechanisms that lead to the positive response exhibited by polycultures.

4. Conclusions

The experiment showed that ACP recycling using polycultures is not only feasible, but could actually increase biomass production while recycling nutrients. Polycultures outperformed even the best single species in terms of coproduct tolerance and productivity when recycling ACP. Due to their superior tolerance to ACP, polycultures could require less dilution or purging in a recycling pathway. These results suggest that ecological interactions could be harnessed to

substantially improve nutrient use efficiency and minimize the production of waste streams that could otherwise contribute to eutrophication.

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Appendix A. Supplementary Methods and Data

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Figure Captions

Figure 1. Pictorial diagram summarizing the experimental design of the mesocosm experiment (upper panel), the aqueous-phase coproduct (ACP) recycling experiment (lower panel), and their relationship. The same set of algal species was used in both studies (center), but only a subset of the species combinations was used for the recycling experiment. In the mesocosm experiment, algae were grown as monocultures and polycultures using fresh medium (1) and biomass from those cultures was processed by hydrothermal liquefaction (HTL) to make ACP and biocrude oil (2). In the recycling experiment, ACP from each source was added to fresh medium at multiple dilutions in culture plates (3) and inoculated with algae in a partial factorial design of ACP source culture and recycler culture (4). The recycling experiment design was repeated a total of four times: two temporal blocks each with two experimental blocks.

Figure 2. Heatmaps of maximum fluorescence and maximum growth rate for all combinations of ACP source cultures and recycler cultures at 0.33%, 1%, and 2% ACP. The color scale represents the mean log response ratio for all viable wells in all four blocks (n=12 in total). For the maximum growth rate heatmaps, black circles indicate that the ACP killed more than 50% of the wells for a combination and black filling indicates that 100% were killed. Additional heatmaps for more dilute ACP concentrations are located in Figure A2.

Figure 3. Box-whisker plots of maximum fluorescence for each recycler culture recycling ACP from all source cultures (left column) and only ACP from the same combination (right column) with colors denoting species richness. Data are compiled from all four blocks. The centerline of each box represents the mean, the edges of the box represent the 25th and 75th

percentiles, the whiskers represent the maximum and minimum values, and filled circles represent outliers more than two times the interquartile distance from the mean. Additional symbols indicate polycultures with higher adjusted means than their component species (overyielding, ∇ and $\blacktriangledown$ for $p < 0.05$) or all six species (transgressive overyielding, $\star$ and $\star$ for $p < 0.05$).

Figure 4. Effect of ACP concentration on viability of recycler cultures when recycling ACP from all source cultures (left) and ACP from the same species combination (right). Colors denote the culture identity within each row.

Figures

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Graphical Abstract