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Tolerance to allelopathic inhibition by free fatty acids in five biofuel candidate microalgae strains

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ABSTRACT

Contaminating organisms (grazers, pathogens, competitors) and self-inhibition by algae-produced allelopathic chemicals are two issues that may limit the productivity of algal cultivation for bioproducts. One potential solution is to identify algal strains that are not affected by allelopathic inhibition even while undesirable organisms are suppressed. Here we used two experiments to test how sensitivity to allelopathy varies across algae. In the first experiment, we tested the sensitivity of five biofuel candidate green algae strains to two allelopathic compounds (i.e., free fatty acids) and found that the degree of inhibition depends strongly on both the species and specific compound. In the second experiment, we exposed one alga (*Chlorella*) to the sterile-filtered medium of each species, and found that the concentration of free fatty acids released into the media predicted *Chlorella*'s growth response. This provides a better understanding of how the production of, and sensitivity to, allelopathic compounds determines algal productivity.

1. Introduction

Algal biomass presents a promising source of renewable biofuels and bioproducts. However, there are many challenges that currently limit the scalability of algae-based industrial bioprocesses. One challenge, which also represents a substantial opportunity, is the management of water chemistry during algal cultivation. This involves, for example, optimizing pH, salinity, and nutrients for algal crop productivity. Specifically, manipulating pH and salinity levels can reduce the risk of major losses to pests and contaminating organisms (Bartley et al., 2014, 2013; Ganuza et al., 2016; Thomas et al., 2017), and addition of exogenous chemicals can effectively control grazers and pathogens (Moreno-Garrido and Cañavate, 2001; Park et al., 2016; Van Ginkel et al., 2015). These solutions will clearly be vital for adaptive management of the complex biological systems open biofuel ponds will inevitably harbor. However, the potential of chemical compounds produced by algae themselves, to function as either algal growth promoters or pest inhibitors, is a relatively novel frontier that has yet to be explored.

Specifically, chemical inhibition of algae by the compounds they produce and release into the water (i.e., allelopathy) at high biomass densities is a key area of concern, as it may limit productivity of mass algal cultivation. Chemical inhibition is particularly important in systems where the culture medium is reused multiple times, allowing allelopathic compounds to accumulate (Loftus and Johnson, 2017). Many of the species that are targets for algal biofuel production due to their high fatty acid content (e.g., *Botryococcus braunii*, *Chlorella* spp.) also leak fatty acids into the water in quantities that could be high enough to cause self-inhibition or toxicity to other algae species. Such inhibition occurs primarily because fatty acids that are released from their glycerol backbone (i.e., free fatty acids) can cause damage to algal plasma membranes, with effects ranging from leakage of intracellular nutrients to complete cellular lysis (Wu et al., 2006). Additional possible mechanisms for toxicity of free fatty acids (FFAs) include increased oxidative stress, as well as inhibition of enzyme activity, nutrient uptake, and DNA synthesis (Casillas-Vargas et al., 2021; Desbois and Smith, 2010). Several case studies have shown the possible effects of FFAs produced by both green algae and cyanobacteria. Though many algal compounds may cause allelopathy, there is particularly strong evidence for inhibitory effects of FFAs in a range of contexts, including controlled laboratory microcosm experiments (Ikawa et al., 1997; McCracken et al., 1980; Pratt and Fong, 1940; Song et al., 2017), growth inhibition in algal photobioreactors (Bosma et al., 2008; Sabia et al.,

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2015), and observations of FFA toxicity in natural ecosystems (Chiang et al., 2004). However, the effects of FFAs are highly variable depending on both the quantity released into the water and the sensitivity of the species being grown (DellaGreca et al., 2010; McCracken et al., 1980). As an example, one alga (*Monodus subterraneus*) was inhibited by palmitic acid at just 0.1 mg L^{-1}; while another (*Chlorella vulgaris*) was only inhibited by palmitic acid at 59 mg L^{-1}, representing a nearly 600-fold difference in sensitivity (Bosma et al., 2008; Wu et al., 2006). Thus, there is evidence for a wide range in species-specific tolerance to individual FFAs; however, this tolerance has only been tested for very few species relevant to industrial cultivation. The primary mechanism by which species have increased tolerance for FFAs is modification of their cell membranes such that they are resistant to their effects. Specifically, resistance to FFA inhibition can arise from traits including increased cell wall thickness, reduced hydrophobicity of cell surfaces, and increased membrane carotenoid levels (Desbois and Smith, 2010).

One approach to managing the release of allelopathic compounds into the water during mass algal cultivation is to design multi-species feedstocks such that the target algae are not sensitive to chemical inhibition by themselves or other algae, and the risk of invasion by grazers, pathogens, and undesired algae is mitigated by allelopathic properties of the cultivation strains (Bacellar Mendes and Vermelho, 2013; Shurin et al., 2013). This strategy is particularly promising for algae-derived free fatty acids, as they can inhibit a broad spectrum of potentially harmful organisms, including bacteria, fungi, weedy algae, and grazing zooplankton (Chiang et al., 2004; Desbois and Smith, 2010; Ruffell et al., 2016). Moreover, extraction of extracellular lipids could significantly enhance the efficiency of algal biofuel production by avoiding costly steps of harvesting, dewatering and cell disruption; this non-destructive process known as “algae milking” is therefore a key area of investigation (Jackson et al., 2020; Kleinert and Griehl, 2020; Liu et al., 2016). Thus, secreted FFAs could function simultaneously to protect the algal crop and also be directly extracted for biofuel feedstock. In such a chemically optimized growth scenario, unprotected and fast growing species (e.g., *Chlorella* or *Nannochloropsis* spp.) can be protected by slower-growing but chemically defended taxa (e.g., *Botryococcus braunii*), in what ecologists call an “associational refuge” (Shurin et al., 2013). The limiting factor for this type of strategic community engineering, however, is identifying strains that are highly productive even in the face of high FFA concentrations. In a different growth scenario where contaminating organisms play a reduced role, for example growing monocultures in controlled photobioreactors, one may want to select a strain that produces very little inhibitory FFAs and/or is robust to self-inhibition by FFAs. In both cases, however, understanding both excretion of FFAs and sensitivity to FFA inhibition would yield important insights into optimizing cultivation.

In this study, we tested the sensitivity of five biofuel candidate microalgal strains to inhibition by free fatty acids, with the goal of identifying highly productive strains that are also resistant to allelopathic inhibition. We tested the growth responses of these algae to pure free fatty acid compounds, oleic acid and linolenic acid, as these specific compounds are secreted by algae and are known to have strongly inhibitory effects on algal growth (Bosma et al., 2008; Chiang et al., 2004; Ikawa et al., 1997; Wu et al., 2006).We then tested the growth of a focal algal species, *Chlorella sorokiniana,* in the filtered medium of all five strains to provide a proof of concept for using algal FFA sensitivity to predict growth responses in recycled medium. These experiments were used to test the following hypotheses: H1) the species used would have significantly different growth responses to the free fatty acids, and H2) that the relative concentrations of FFAs produced by each species would predict the growth response of *Chlorella sorokiniana*.

2. Methods

2.1. Algal strains and culture maintenance

The strains used in this study were: *Ankistrodesmus falcatus* (UTEX B749), *Botryococcus braunii* (UTEX 2441), *Chlorella sorokiniana* (UTEX 2805), *Scenedesmus acuminatus* (SAG 38.81), and *Selenastrum capricornutum* (UTEX 1648); all are referred to hereafter by their genus names. These strains were chosen due to: their demonstrated potential for biocrude production in both mono- and polycultures (Godwin et al., 2017; Narwani et al., 2016); the documented variation in production of free fatty acids across these species and; because when grown in pairwise combination, the included species show a wide range of interactions from stimulation to inhibition (Venail et al., 2014), which could potentially be due to allelopathic interactions. All strains were maintained in COMBO algal growth medium at pH 7.5 (Kilham et al., 1998), at 20 °C, and ca. 100 μmol photons m^{-2} s^{-1} until use for the experiment.

2.2. Free fatty acid inhibition assays

Oleic acid (C18:1n9) and linolenic acid (C18:3n3) were purchased from Fisher Scientific (cat no. AC270290050 and AC302825000, respectively). Following methods of previous studies (DellaGreca et al., 2010; McCracken et al., 1980), a non-toxic solvent was used to ensure that the FFAs were soluble in the medium. Specifically, each fatty acid was added to a solution of DMSO; we then combined this solution of dissolved FFAs with COMBO medium such that the final concentration of DMSO was 0.2 % v/v, a concentration that does not affect algae (Hu et al., 2017), and adjusted the pH to 7.5. Each fatty acid was added in various amounts to provide a gradient of eight FFA concentrations: 0, 0.5, 1, 2, 5, 10, 20, and 50 mg L^{-1}. These values were chosen based on previous work showing significant FFA inhibition at concentrations ranging from <1 to 26 mg L^{-1}, depending on both the species and the specific FFA (Bosma et al., 2008; DellaGreca et al., 2010; Wu et al., 2006). These FFA solutions were then distributed into 48-well plates for the growth inhibition assay, with 0.8 mL culture solution per well. Each well was inoculated with 10^4 cells mL^{-1}, after which, well plates were sealed with sterile gas-permeable Breathe-Easy® membranes. The aforementioned design resulted in a total of 80 unique treatments (5 species × 8 concentrations × 2 FFAs), each of which was replicated 6 times, for a total of 480 experimental units. All culture plates were then cultivated in a Percival AL-41 algal growth chamber (20 °C, ca. 100 μmol photons m^{-2} s^{-1}, 16:8 light:dark) with a rotary shaker table (120 rpm) to keep cultures suspended. Growth was measured daily for 10-days via in vivo chlorophyll-a fluorescence using a BioTek Synergy H1 plate reader and excitation/emission wavelengths of 460/685. Estimates of growth data were calculated using the first 4 days of fluorescence measurements, as this initial 4-day period best captured the exponential growth phase directly following the FFA manipulations.

2.3. Growth assays with recycled medium

Chlorella was chosen as the focal species for this experiment due to its rapid growth, high lipid productivity, and demonstrated importance in the biofuel industry. To assess how the overall chemical fingerprint of each strain affects growth of *Chlorella*, all five strains were grown in 1-L culture bottles until they reached stationary phase. Each culture was then filtered through 0.22 μm Stericup® filters to obtain sterile filtrate. To ensure nutrients were still in excess in the filtrate, COMBO nutrients were added to the filtrate to increase nutrient levels comparable to those in standard COMBO medium, and pH was adjusted to 7.5. The used culture medium filtrate of each species was then distributed into 48-well plates and inoculated with *Chlorella* at 10^4 cells mL^{-1}. For this experiment, there were 8 replicates per treatment. Subsequent growth measurements and culture conditions were identical to those of the FFA

inhibition test described in Section 2.2.

2.4. Estimation of relative FFA concentrations in used culture medium

The same culture filtrate used for the *Chlorella* growth assays (Section 2.3) was used to estimate FFA concentrations in the medium (i.e., samples were obtained from the same 1-L culture bottle of each species). This 0.22 μm-filtered culture medium was frozen at −20 °C until it was used for extraction and analysis. At this point, liquid-liquid extraction, with dichloromethane (DCM) as a solvent, was used to extract the nonpolar fraction of compounds in the media. The DCM extract was then concentrated using a rotary evaporator. The method of Levine et al. (2012) was then followed for the transesterification and quantification of fatty acids. Briefly, this method extracts and converts fatty acids to fatty acid methyl esters (FAMEs) by acid-catalyzed transesterification. FAMEs were quantified using gas chromatography with a flame ionization detector (GC-FID). Results are reported as peak area relative to the peak area of the internal standard tricosanoic acid (C23:0).

2.5. Statistical analysis

Growth rates for all algal populations (based on fluorescence measurements over time) were estimated using the function "all_growthmodels" in the R package "growthrates" (v. 0.8.2, Petzoldt, 2019), with the growth model set to "grow_exponential" (i.e., the functions required to fit a standard exponential population growth model). Effects of FFA concentrations on growth rates were tested by fitting nonlinear models to the data using the "drm" function of the "drc" package (v. 3.0–1) in R, which is designed to carry out standard dose-response curve analysis (Ritz et al., 2015). Specifically, the 3-parameter log-logistic model was used to estimate values of EC50, the concentration at which growth is reduced by 50 % relative to the control. The 95 % confidence intervals of the EC50 values were then used to assess differences in sensitivities to free fatty acids based on species and the distinct compounds. ANOVAs were used to test for differences in *Chlorella* growth in media of different species. All analyses were carried out in R version 4.0.3 (R Core Team, 2020).

3. Results and discussion

3.1. Effects of free fatty acids on algal growth

There were significant negative effects of free fatty acids (FFAs) on algal growth, but these effects depended strongly on the specific FFA compounds, the concentration, and the algal species (Table 1, Figs. 1, 2). For example, while all species maintained positive growth rates when exposed to oleic acid, increasing concentrations of the fatty acid led to a reduction in growth rates for all species except *Botryococcus*. Linolenic acid, however, caused severe growth inhibition of all species when at higher concentrations (>10 mg L^{-1}). This indicates the strong differences in inhibitory effects caused by the two fatty acids. For example, *Chlorella* had only a mild and non-significant decline in growth rate with greater concentrations of oleic acid addition (Fig. 2 left panel) and a very high EC50 value (Table 1); this indicates a very low sensitivity to oleic acid. Under linolenic acid addition, however, *Chlorella* growth ceased entirely at FFA concentrations over 2 mg L^{-1} (Fig. 2, right panel), indicating that linolenic acid is much more inhibitory than oleic acid (i.e., the EC50 value for *Chlorella* was 100-fold lower for linolenic than oleic acid, Table 1). The data also indicate differential sensitivity to FFAs for different species as shown by their EC50 values (Table 1). For example, *Ankistrodesmus* (EC50 = 16.11) was more sensitive to oleic acid than *Chlorella*, (EC50 = 406), while *Chlorella* (EC50 = 4.17) was significantly more sensitive to linolenic acid than *Ankistrodesmus* (EC50 = 10.40). This supports our first hypothesis, that different species would have significantly different responses to allelopathic FFA compounds.

It is also worth noting that some strains that initially had strong inhibition were able to eventually start growing after day 3, as seen in the growth curves in Fig. 1 where fluorescence does not increase until later in the experiment. This suggests either that (a) there was differential susceptibility of cells in the algal populations that allowed the surviving cells to regrow in some treatments, and/or (b) that FFAs degraded over this time, allowing subsequent growth (e.g., see Fig. 1; *Selenastrum* at 10 and 20 mg L^{-1} linolenic acid). In other cases, however, the cultures did not recover, indicating that cell viability was insufficient for recovery of the algal population (e.g., see Fig. 1; *Selenastrum* at 50 mg L^{-1} linolenic acid).

The range of FFA concentrations at which inhibition occurred (see EC50 values in Table 1) aligns with previous observations showing inhibition between 6.5 and 26 mg L^{-1} (DellaGreca et al., 2010; Wu et al., 2006). However, the relative strengths of oleic and linoleic acids differ in our study compared to results of Bosma et al. (2008), who found oleic acid to be more acutely toxic than linoleic acid, with a 50 % inhibition of growth occurring at <1 mg L^{-1} oleic acid. Chiang et al. (2004) found linolenic acid to be more toxic to zooplankton than oleic acid, but that inhibitory effects of the two compounds were equivalent for phytoplankton. These observed differences in FFA sensitivity between algae and their consumers suggest that taxon-specific inhibitory effects may be further exploited to enhance resistance of industrial algal cultivation to grazing pests. For example, species like *Scenedesmus* had relatively high tolerance to FFAs in our study. Future work should test whether *Scenedesmus* and other FFA-tolerant algae can indeed grow well at FFA levels that would inhibit grazers or pathogens. Additionally, future work may focus on directed evolution and/or gene editing approaches to enhance the tolerance of industrial algae strains to FFAs and other algae-derived inhibitory compounds.

To our knowledge, there are no published values of free fatty acids in open ponds used for algal biofuel or bioproduct cultivation. Concentrations in natural systems (e.g., Lake Taihu) are much lower than those reported in laboratory microcosms, yet allelopathic effects on competing algae have still been attributed to these compounds (Song et al., 2017).

Table 1
Parameter estimates for EC50 values (i.e., the FFA concentration causing a 50 % reduction in growth rate), their standard error (SE), and 95 % confidence intervals (CI). Bold values for EC50 denote significantly inhibitory effects of FFAs with α set to 0.05; non-overlapping 95 % CIs indicate significant differences in FFA sensitivity among species.

Species	FFA compound	EC50	SE	p	Lower 95 % CI	Upper 95 % CI
Ankistrodesmus	Oleic acid (C18:1n9)	**16.11**	6.78	**0.022**	2.44	29.77
Botryococcus	Oleic acid (C18:1n9)	4331.54	1.8×10^5	0.981	-3.5×10^5	3.6×10^5
Chlorella	Oleic acid (C18:1n9)	406.33	218.78	0.070	−34.32	846.97
Scenedesmus	Oleic acid (C18:1n9)	123.09	71.27	0.091	−20.47	266.64
Selenastrum	Oleic acid (C18:1n9)	**26.56**	2.39	$\mathbf{1.7 \times 10^{-14}}$	21.76	31.37
Ankistrodesmus	Linolenic acid (C18:3n3)	**10.40**	1.26	$\mathbf{1.4 \times 10^{-10}}$	7.87	12.94
Botryococcus	Linolenic acid (C18:3n3)	5.83	10.25	0.573	−14.83	26.48
Chlorella	Linolenic acid (C18:3n3)	**4.17**	1.41	**0.005**	1.33	7.01
Scenedesmus	Linolenic acid (C18:3n3)	**10.84**	4.02	**0.010**	2.75	18.94
Selenastrum	Linolenic acid (C18:3n3)	**9.88**	0.20	$\mathbf{2.1 \times 10^{-41}}$	9.48	10.28

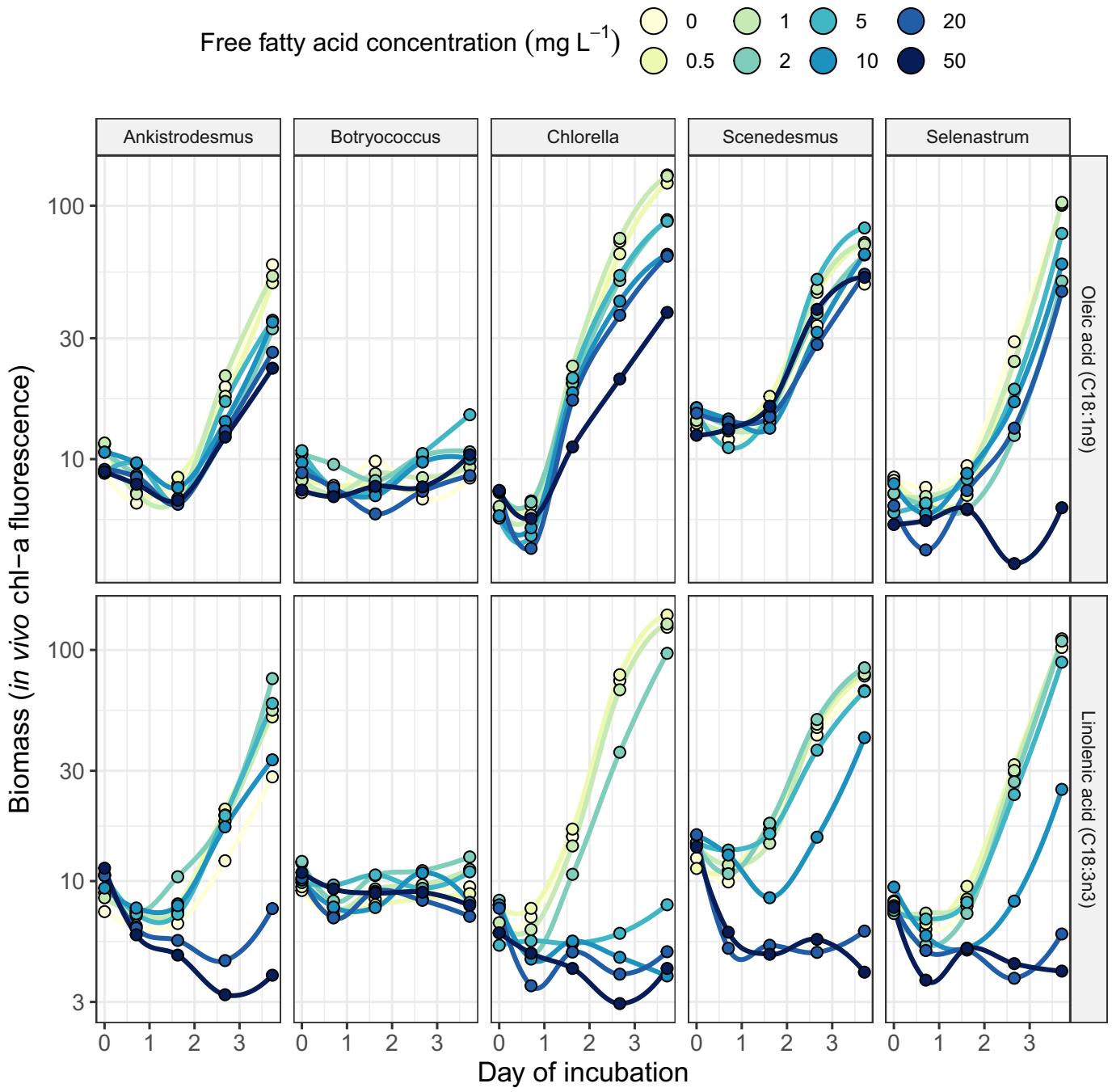

Fig. 1. Growth over time of the five biofuel candidate species under a gradient of free fatty acid (FFA) concentrations. Growth responses varied depending on the species, the FFA concentration, and the specific type of FFA. The y-axis represents chl-a biomass in terms of relative fluorescence units (RFU). Lines represent loess fits.

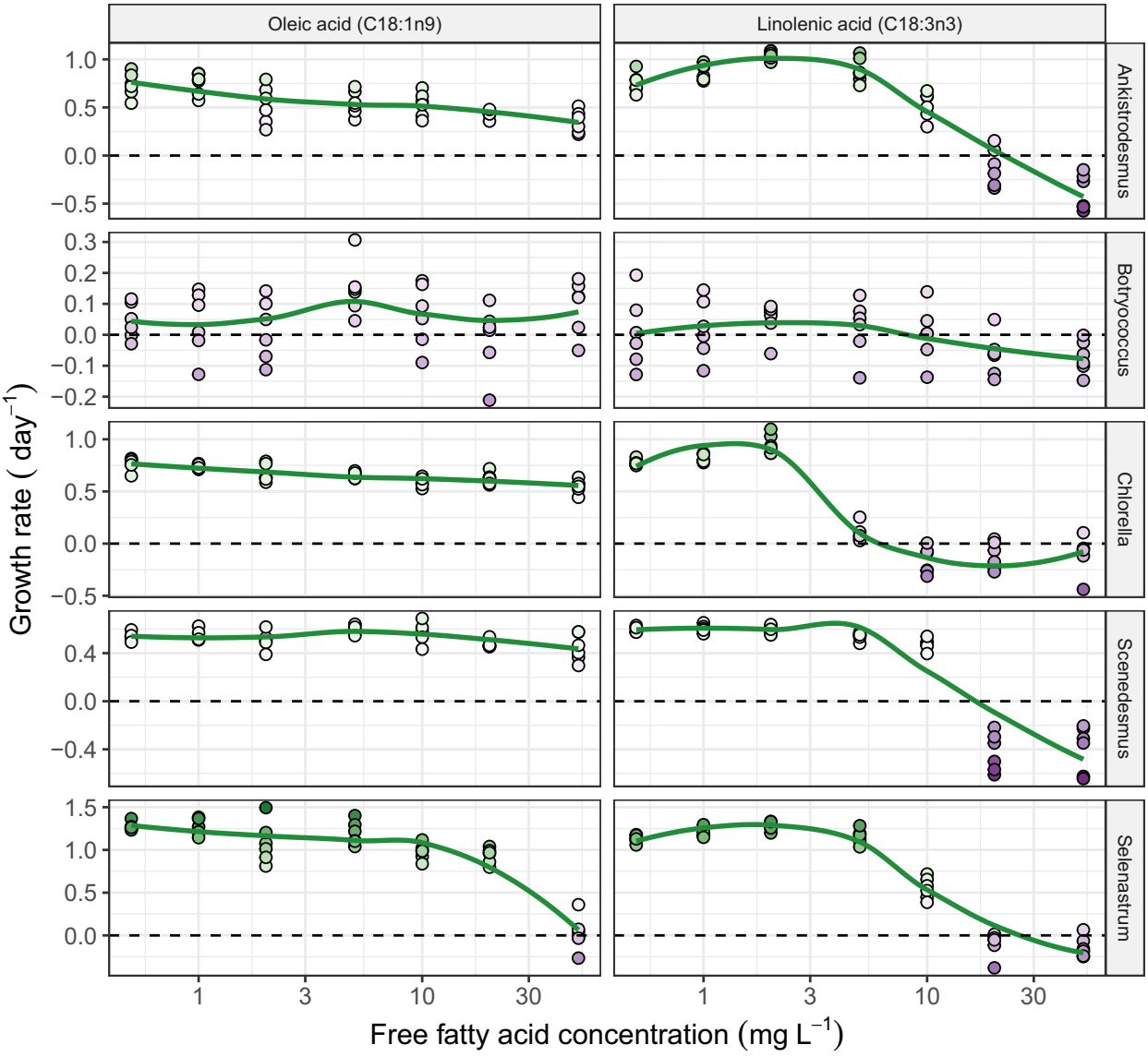

Fig. 2. Growth rates of the five biofuel candidate species with a gradient of free fatty acid (FFA) concentrations. Sensitivities to these allelopathic compounds varied depending on the species, the FFA concentration, and the specific type of FFA. Lines represent loess fits, and color corresponds to growth rates.

A better understanding of the free fatty acid concentrations in photobioreactors and open ponds will, of course, provide information about the extent to which inhibitory effects occur in industrial conditions.

3.2. Effects of recycled culture filtrate on Chlorella growth

There was significant variation in the effect of different species' filtrate on *Chlorella*'s growth patterns (Fig. 3), as well as variation in FFA production across species (Fig. 4). Our analysis of the relative concentrations of FFAs in the medium of the species assessed confirms that *Botryococcus* produced the most FFAs, followed closely by *Chlorella*, and then *Ankistrodesmus*, *Scenedesmus*, and *Selenastrum* (Fig. 4). This aligns well with previous observations of FFA concentrations in which *Botryococcus* and *Chlorella* produce the greatest concentrations of soluble free fatty acids (Chiang et al., 2004).

Additionally, the relative amount of FFAs released into the medium was a significant predictor of both the growth rate and the biomass of *Chlorella* cultures (Fig. 4), in support of our second hypothesis. *Botryococcus* medium elicited the most negative effects on *Chlorella* growth rate and biomass yield, followed by the medium of *Chlorella* itself. On the other hand, *Ankistrodesmus* and *Selenastrum* both allowed *Chlorella* to grow significantly better than it did in its own recycled culture medium (ANOVA for growth rate: $F_{4,\ 35} = 41.8$, $p = 7 \times 10^{-13}$; ANOVA for biomass: $F_{4,\ 35} = 110.9$, $p = 2 \times 10^{-16}$). *Scenedesmus* culture medium neither enhanced nor inhibited *Chlorella* growth rates, but did enhance *Chlorella* biomass production. In terms of the relative magnitude of the effect of growing in recycled filtrate: when *Chlorella* was grown in the filtrate of *Ankistrodesmus*, it had 33 % faster growth and 69 % higher final biomass relative to when it was grown in its own recycled medium. Conversely, *Chlorella* grown in *Botryococcus* medium had 37 % slower growth and 26 % lower final biomass.

The above results provide two insights: 1) that *Chlorella* and *Botryococcus* may not be an optimal choice for polyculture, as the strong inhibition of *Chlorella* by exudates from *Botryococcus* may reduce biomass productivity; and 2) that *Chlorella* grown in the filtrate of other species (e.g., *Ankistrodesmus*, *Selenastrum*) may exhibit overyielding when compared to growth in its own medium. This implies potential benefits of either using these species in polyculture with *Chlorella*, or of a crop rotation paradigm designed to take advantage of productivity benefits from recycling medium of compatible species. Such a crop rotation strategy has been suggested previously as an approach to mitigate pest infestations (Carney and Lane, 2014; Shurin et al., 2013;

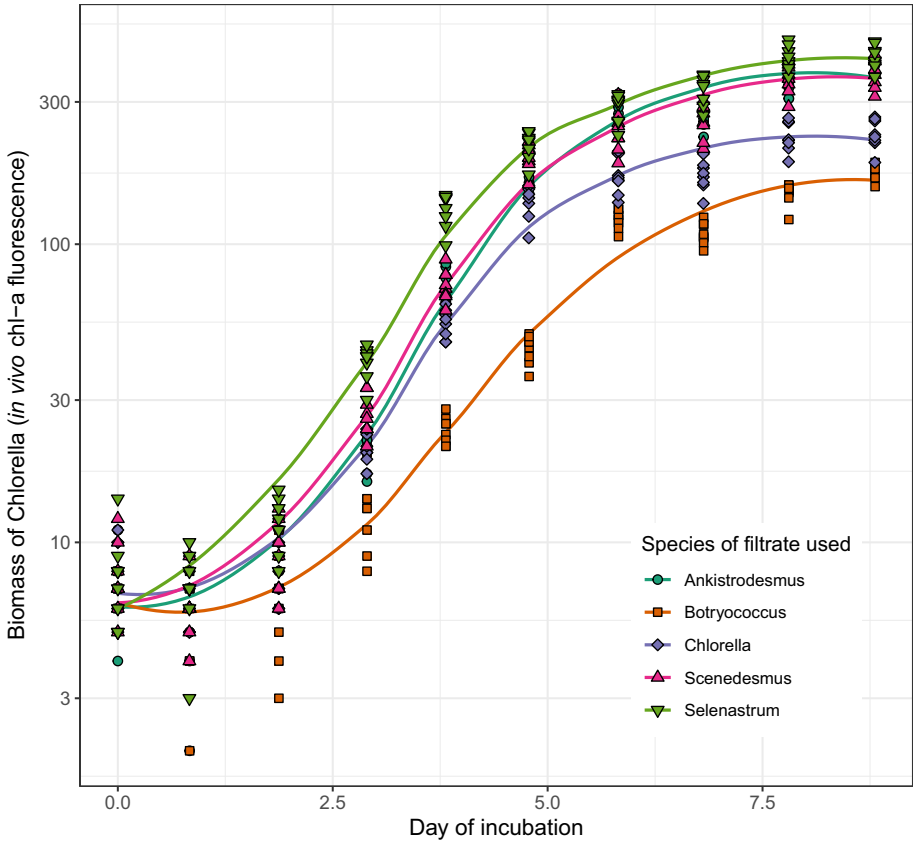

Fig. 3. Growth of *Chlorella sorokiniana* in the filtered and nutrient-amended culture medium of the five algae species. *Chlorella* growth rate and biomass both varied significantly depending on the species which the recycled medium originated from. Shapes and colors represent the five distinct algal taxa which the filtered medium originated from. The y-axis represents chl-a biomass in terms of relative fluorescence units (RFU). Lines represent loess fits.

Smith et al., 2015). Our data suggest this approach has potential for directly boosting algal production as well, regardless of grazer or pathogen contamination. On the other hand, the synthesis by Loftus and Johnson (2017) shows an average negative effect of growing algae in the recycled medium of a different species. Results from their meta-analysis therefore suggest that finding appropriate species combinations based on compatibility of their chemically-mediated traits would be of key importance to a targeted crop rotation strategy.

Although our study and several others clearly show that FFAs significantly impact algal productivity, there are many additional compounds that may inhibit or stimulate algal growth, as algal exometabolomes can be comprised of hundreds of unique chemical features (Becker et al., 2014; Brisson et al., 2021). For example, recent work shows that diverse exometabolites including prostaglandins, aldehydes, dodecanedioic acid, L-histidinal, tiliacorine, dimethylsulfoniopropionate (DMSP), and others can also play important roles in allelopathy among phytoplankton (Apostolopoulou et al., 2022; Brisson et al., 2021). Algae can also produce and excrete phytohormones like indole-3-acetic acid, which may stimulate growth of other species (Liu et al., 2016; Mazur et al., 2001) The high dimensionality of algal chemical exudates therefore poses a potential challenge; however, the richness of exometabolites we can extract from algae cultures also presents a substantial opportunity for industrial utilization (Liu et al., 2016).

Although polycultures provide substantial benefits for algal cultivation (Godwin et al., 2018; Narwani et al., 2016; Newby et al., 2016; Thomas et al., 2019), they rarely outperform their constituent monocultures with respect to biomass yields (Schmidtke et al., 2010; Shurin et al., 2014). Allelopathy is one plausible mechanism for such underyielding; however, this has not been explicitly measured in any biodiversity-productivity studies to our knowledge. Conversely, variation in the chemical fingerprint of microalgae may also help explain why certain polycultures exhibit overyielding (i.e., if compounds in the medium of another species facilitate enhanced growth). Recent work shows that building algal polycultures based on their functional trait differences can increase the chances of finding highly productive species pairs (Mandal and Corcoran, 2022); including functional traits related to algal chemical fingerprints may further enhance our ability to construct productive synthetic algal communities. Our results therefore suggest that studying variation in the production of, and sensitivity to, allelopathic and stimulatory compounds, may provide important insights regarding the utility of polycultures for mass algal cultivation. Future work should expand on this framework to assess sensitivities of more algal species, as well as pests that invade industrial algae facilities, in order to provide further insights into enhancing algal productivity via optimizing pond chemistry.

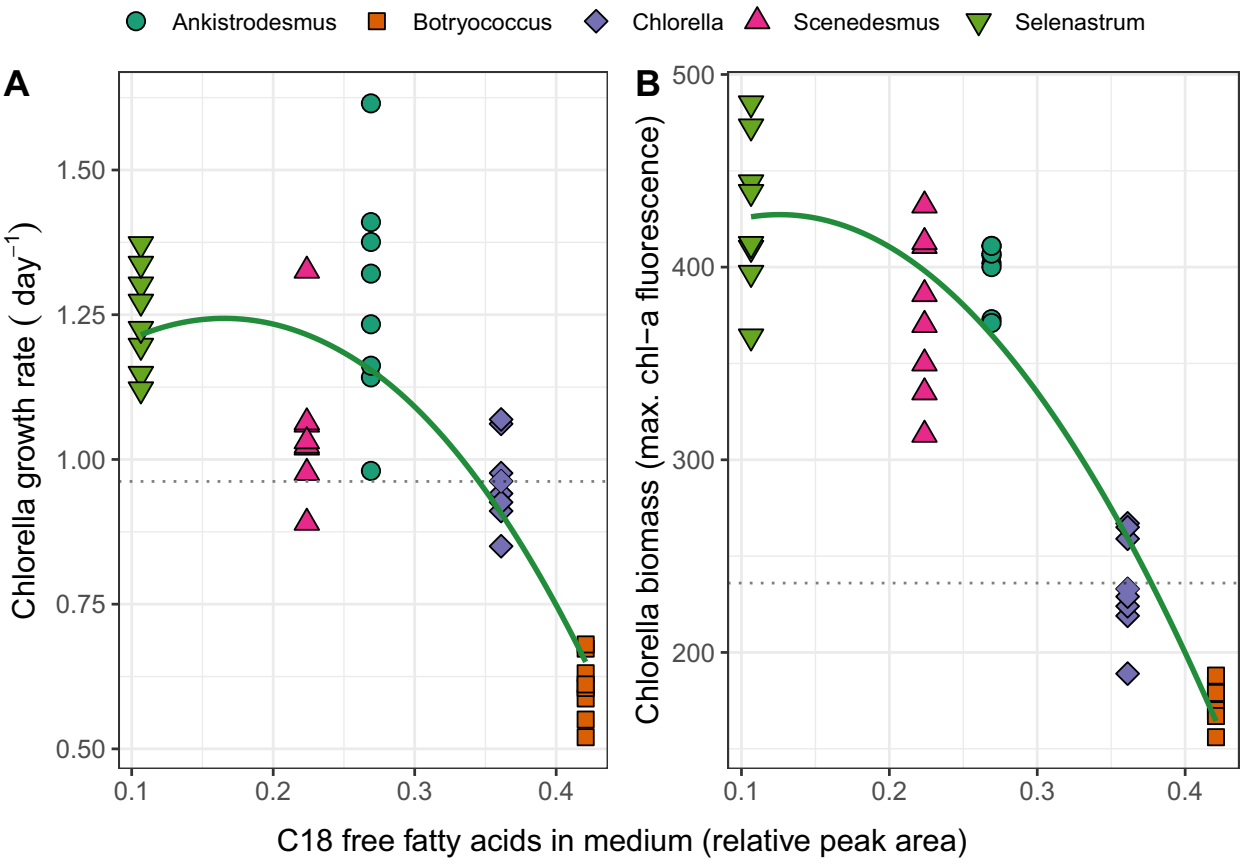

Fig. 4. Effects of the relative amount of 18-carbon free fatty acids in the medium of each species (in terms of GC-FID relative peak area) on (A) growth rate and (B) biomass of *Chlorella sorokiniana*. Shapes and colors represent the five distinct algal taxa which the filtered medium originated from. Horizontal dashed lines represent average growth rate and biomass values for *Chlorella* grown on its own filtered medium. The y-axis in (B) represents chl-a biomass in terms of relative fluorescence units (RFU). Lines represent loess fits.

4. Conclusions

We showed that 5 biofuel candidate green algae strains have very distinct sensitivities to free fatty acids (FFAs), with estimated EC50 values ranging from 4 to >4000 mg L^{-1} depending on both the species and the fatty acid compound, and that the amount of FFAs produced varied strongly across species. We further show that differences in exometabolites across species can cause anywhere from a 37 % decrease to a 33 % increase in *Chlorella* growth rates. This study provides a proof-of-concept for designing algal cultures by understanding their chemically mediated interactions in both mono- and polycultures.

CRediT authorship contribution statement

Patrick K. Thomas: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **David C. Hietala:** Methodology, Investigation, Writing – review & editing. **Bradley J. Cardinale:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data produced in this work, as well as annotated R code used for all analyses, are available on the Mendeley Data repository (https://doi.org/10.17632/vpfkpp42bh.1).

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