



Botryococcus braunii reduces algal grazing losses to *Daphnia* and *Poteroiochromonas* through both chemical and physical interference

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Abstract

Crop protection from algal grazers is a key area of concern, as grazing zooplankton and flagellates can decimate microalgal crops and impede economic viability of cultivation for biofuels and bioproducts. Inhibition of grazing by chemical and physical interference is one promising solution; however, there have been few empirical tests of this approach that use defense traits innate to algal crop species. *Botryococcus braunii* is of particular interest because a) it excretes high levels of hydrocarbons and exopolysaccharides and b) forms colonies and possesses chemical defenses. Here we conduct a controlled laboratory experiment to test whether *B. braunii* can mitigate losses to grazing by two distinct grazers, *Daphnia magna* and *Poteroiochromonas malhamensis*, due to both chemical inhibition and physical interference linked to large/inedible colonies. We show that chemical and physical defenses interactively reduce the total effect of grazing, thus significantly increasing the biomass and growth rates of cultures of *B. braunii* and *Nannochloropsis limnetica* when either grazer is present. We also find that *B. braunii* medium enhances the growth of *N. limnetica*. Our study demonstrates how community engineering can identify synergies arising from algal co-cultivation (e.g., by using industrially relevant strains for crop protection). While our lab study serves as a proof-of-concept, future research should test this strategy at pilot scale; if successful, such ecological discoveries may help to reduce the costs of large-scale deployment of algal cultivation for sustainable foods, fuels, bioproducts (e.g., bioplastics), and carbon capture.

Keywords Chemical ecology · Pest management · Synthetic ecology · Algal bioproducts · Algae milking · Allelopathy

Introduction

Protecting microalgal crops from losses to grazers, parasites, and pathogens is a key area of concern, as these organisms can cause rapid pond crashes that threaten the sustainability and economic viability of algae cultivation for bioproducts (Day et al. 2017; Lane 2022). In general, the most productive, fastest-growing microalgae (i.e., common targets for bioproducts) have small cell sizes which are optimal for maximizing resource uptake and thus growth rates, but are also highly susceptible to grazing by zooplankton including

flagellates, rotifers, and cladocerans (Hillebrand et al. 2022). Specifically, taxa including *Chlorella*, *Nannochloropsis*, and *Scenedesmus* are promising due to their high productivity of lipids, proteins, and carbohydrates which can be used for a variety of products (e.g., animal feed, omega-3 fatty acid supplements, biofuels, bioplastics). However, they are also highly susceptible to grazing when grown in unprotected monocultures (Day et al. 2017; Ma et al. 2017; Thomas et al. 2017, 2019); this can create a trade-off between baseline productivity and grazing resistance capacity. There are several described strategies to overcome this trade-off between maximum productivity and increased risk of pond crash due to grazing or parasitism. Specifically, grazers and fungi can be controlled via external inputs like pesticides and fungicides (McBride et al. 2014; Van Ginkel et al. 2020; McGowen et al. 2023) or can be managed by applying pH, ammonia, or salinity changes that inhibit pests but not algae (Day et al. 2017; Ma et al. 2017; Thomas et al. 2017). However, using one of these solutions in isolation is unlikely to

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exhibit long-term efficacy against all aquatic grazers and parasites, because biological pest populations can respond adaptively, and their communities are dynamic. This calls for using complementary adaptive management strategies to stabilize large-scale production. Ideally, these interventions will not increase operational costs, energy input, or risk of pesticide release to the environment.

One approach with the potential to stabilize annual productivity is to use designed polycultures, such that different algae species or strains with complementary traits can overcome trade-offs faced by monocultures (Shurin et al. 2013; Kazamia et al. 2014; Nalley et al. 2014; Newby et al. 2016; Thomas et al. 2019), thus simultaneously maintaining baseline productivity and preventing pond crash. This can take a variety of forms, including, e.g., using chemically-defended strains (Bacellar Mendes and Vermelho 2013) or large-celled/colonial strains (Corcoran and Boeing 2012) to act as an “associational refuge” capable of protecting undefended but faster-growing strains (Shurin et al. 2013). Despite the promise of using chemically/physically defended strains for crop protection, we currently lack experimental evidence for the efficacy of this approach using industrially relevant strains.

One species of intensive focus in the algae industry is *Botryococcus braunii* due to its high production of extracellular hydrocarbons which can be non-destructively extracted for liquid biofuels (i.e., “algae milking”) (Moheimani et al. 2013; Griehl et al. 2015; Ennaceri et al. 2023) as well as its high excretion of exopolysaccharides in certain strains (Fernandes et al. 1989; Blifernez-Klassen et al. 2018). However, *B. braunii* may also have untapped potential in its role for protecting undefended algal crops. Shurin et al. (2013) indeed suggested this possibility for crop protection due to *B. braunii*’s ability to chemically inhibit other zooplankton and algae by secreting inhibitory levels of free fatty acids into the medium (Chiang et al. 2004), as well as its large colonies offering physical protection from grazing. Therefore, an important opportunity in designing robust algal communities is to test whether *B. braunii* can inhibit grazers without inhibiting undefended target algae (e.g., *Nannochloropsis*).

In this study we tested whether *B. braunii* inhibits two common and important freshwater grazers and one algal species via both chemical and physical interference. We used *Daphnia magna* and *Poterioochromonas malhamensis* as our two taxonomically and functionally distinct grazer species, and *Nannochloropsis limnetica* as a small (< 4 µm diameter), fast-growing target alga for co-culture with *B. braunii*. *Daphnia magna* was chosen as a representative large-bodied (length < 1 mm as juvenile; 1–5 mm as adult), filter-feeding arthropod zooplankton grazer with a larger prey size, while the golden alga *Poterioochromonas* was chosen as a representative small-celled (< 10 µm diameter) flagellate with a smaller prey size that consumes cells via phagocytosis; both

are common worldwide and are capable of strong top-down control of algal biomass (Day et al. 2017; Ma et al. 2023). We experimentally tested the following hypotheses: H1) *B. braunii* reduces algal biomass losses due to grazing via a combination of chemical and physical interference; and H2) *B. braunii* does not chemically inhibit *N. limnetica* growth.

Materials and methods

Algae and zooplankton cultures

The strain of *Botryococcus braunii* used in this experiment was isolated from a bloom in Eawag’s experimental ponds (Dübendorf, Switzerland, 47°24′18.2″ N 8°36′31.7″ E) in April 2023. According to partial 18S sequence matches it belongs to the A-clade and is closely related to previously described strains including, e.g., OIT-560, OIT-284, and CCAP 807/1. It also exhibits traits similar to some B-clade strains of *B. braunii* such as flotation, which is linked to high hydrocarbon content. *Poterioochromonas malhamensis* (CCAP 933/1C) and *Nannochloropsis limnetica* (SAG 18.99) were obtained from culture collections. *Daphnia magna* was obtained from a clonal culture maintained for several years at Eawag and was fed *N. limnetica* as a food source in the weeks leading up to the experiment. Prior to the experiment, all algae and grazer cultures were maintained at 20 °C in COMBO medium with double the concentration of all nutrients described in the original medium recipe (Kilham et al. 1998) to allow for higher algal biomass density (hereafter referred to as 2X COMBO).

Experimental design

For each of the three grazer treatments (grazer-free control, *P. malhamensis*, *D. magna*), there were four *B. braunii* treatments: 1) control (no *B. braunii*); 2) *B. braunii* culture filtrate only, representing only chemical interference; 3) *B. braunii* colonies only, representing primarily physical interference; and 4) *B. braunii* colonies and medium together, representing a combination of both chemical and physical interference of grazing. Treatment 2 (*B. braunii* filtrate only) was made by collecting 0.2-µm filtered medium (i.e., filtrate from Treatment 3) and individually adding 2X COMBO stock nutrients and vitamins to ensure nutrients were initially in excess. Treatment 3 (*B. braunii* colonies) was made by filtering *B. braunii* culture through a 0.2-µm PES Stericup filter and resuspending colonies in fresh 2X COMBO medium. Treatment 4 (colonies and medium) was created by adding 2X COMBO stock nutrients and vitamins directly to a dense culture of *B. braunii*. Specifically, for Treatments 3 and 4, *B. braunii* from stationary phase cultures was added at a constant density of 1498 ± 264 SD relative fluorescence units

(RFU), equal to 1.1×10^4 colonies mL^{-1} . It should be noted that Treatment 3 (colonies only) isolates the effects of physical interference only earlier in the experiment, while later it likely also represents chemical interference as *B. braunii* metabolites accumulate in the medium (i.e., previous work finds that *B. braunii* extracellular products are relatively low in the first week of growth and peak after around 15 days (Blifernez-Klassen et al. 2018)). Treatments 2 and 4 are meant to represent the full chemical fingerprint of *B. braunii* medium. This chemical fingerprint includes exuded metabolites of *B. braunii* that have been previously described, including free fatty acids, hydrocarbons, and exopolysaccharides (Chiang et al. 2004; Blifernez-Klassen et al. 2018; Ennaceri et al. 2023); it also includes changes in inorganic chemical parameters such as pH. While it was not feasible to measure pH in the experimental units, the pH of stationary phase *B. braunii* culture medium used in the experiment was near 9 (compared to pH 8 in the control COMBO medium). We thus cannot entirely rule out pH effects, but we assume that this difference in pH has minimal influence and that chemical effects are driven largely by exuded metabolites.

Aliquots from a stationary phase *N. limnetica* culture were added at equal densities (153 ± 15 SD RFU, equal to 3.1×10^5 cells mL^{-1}) to each of the four *B. braunii* treatments. These mixtures were dispensed into sterile 24-well plates with a volume of 2 mL per well, randomized order in well plates, and 6 replicates per treatment (except for 1 rep. lost in the *P. malhamensis* treatment), in Infors incubators set to 20 °C, rotating at 100 rpm, with 12h:12h light:dark cycle and 104 ± 3.2 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Juvenile *D. magna* of similar age and size were used for the experiment, with one individual per well; 100 μL of stationary phase *P. malhamensis* culture was added to each well (i.e., a 5% v/v dilution yielding 4.5×10^4 cells mL^{-1}). These grazer densities were chosen as pre-trials showed that they were sufficient to exert control on biomass. Growth was tracked using in vivo chlorophyll fluorescence (recorded in RFU) as a proxy for total algal biomass, with excitation/emission at 460/685 nm respectively, using a Biotek Cytation 5 plate reader. The maximum fluorescence value achieved over time was used as a metric for the highest total algal biomass achieved during the experiment for each experimental unit. Note that such fluorescence values capture chlorophyll present in *N. limnetica* as well as *B. braunii* and, to a lesser extent, *P. malhamensis*, which also yields a relatively weak chl-a fluorescence signal due to its lower chlorophyll content. As chlorophyll content can change with environmental conditions or cell health, fluorescence may not linearly relate to cell counts or biomass; however, we interpret fluorescence as indicative of total photosynthetically active biomass, which may be more directly related to the density of live and metabolically active algae. The experiment was conducted for

a total of 18 days. We report algal growth for the first 3 days for *D. magna* treatments, as *D. magna* survival, which was recorded daily in experimental units via microscopy, was high during this period (96%) reflecting an effective grazing treatment and was sufficient to observe differences across treatments (Fig. S1). However, *D. magna* survival gradually declined after day 3 across all *B. braunii* treatments, thus reducing efficacy of the grazing treatment. We report 18 days of growth for the other grazing treatments. On day 18, Lugol-preserved samples were collected for estimating final cell densities. From these preserved samples, cell counts were obtained by imaging with a Leica DMi8 microscope; cells were then enumerated in ImageJ v1.52a. Final cell counts were obtained for the target algae species, *N. limnetica*, and for *P. malhamensis*. Growth rates were calculated as $\ln(\text{RFU}_{\text{day3}}/\text{RFU}_{\text{day0}})/3$ to capture the initial change in fluorescence during nutrient-replete exponential growth over the first 3 days (i.e., while nutrients are in excess in the first few days, we are more certain that effects are due to physical/chemical defenses and not due to differences in nutrient levels). Effects of grazers on growth rate and biomass were interpreted in relation to the respective *B. braunii* treatment with grazers absent, as this allows us to more directly quantify the net effect of grazing on total algal biomass. For example, the effect of *Daphnia* on *N. limnetica* biomass (as % change) when grown in *B. braunii* filtrate is:

$$\text{Effect (\% Change)} = \frac{B_D - B_0}{B_0}$$

where B_D = biomass with *Daphnia* and in *B. braunii* filtrate and B_0 = biomass with no grazer and in *B. braunii* filtrate.

2-way ANOVAs were used to test for the individual and interactive effects of chemical and physical interference on the growth rate and biomass of all treatments; detailed ANOVA results are in Table S1; Tukey HSD was used to test for differences between individual treatments. Statistics were performed in R version 4.2.2 (R Core Team 2020). All data and code are available on Zenodo (<https://doi.org/10.5281/zenodo.13237033>).

Results

Botryococcus braunii inhibits grazing by *Daphnia magna* through both chemical and physical interference

In the control treatment with no *B. braunii* present, *D. magna* rapidly reduced *N. limnetica* biomass (Fig. 1A) over the first three days, with a mean 62% decrease in max. chl-a fluorescence (Fig. 2A; 74.4% loss if one outlier is excluded).

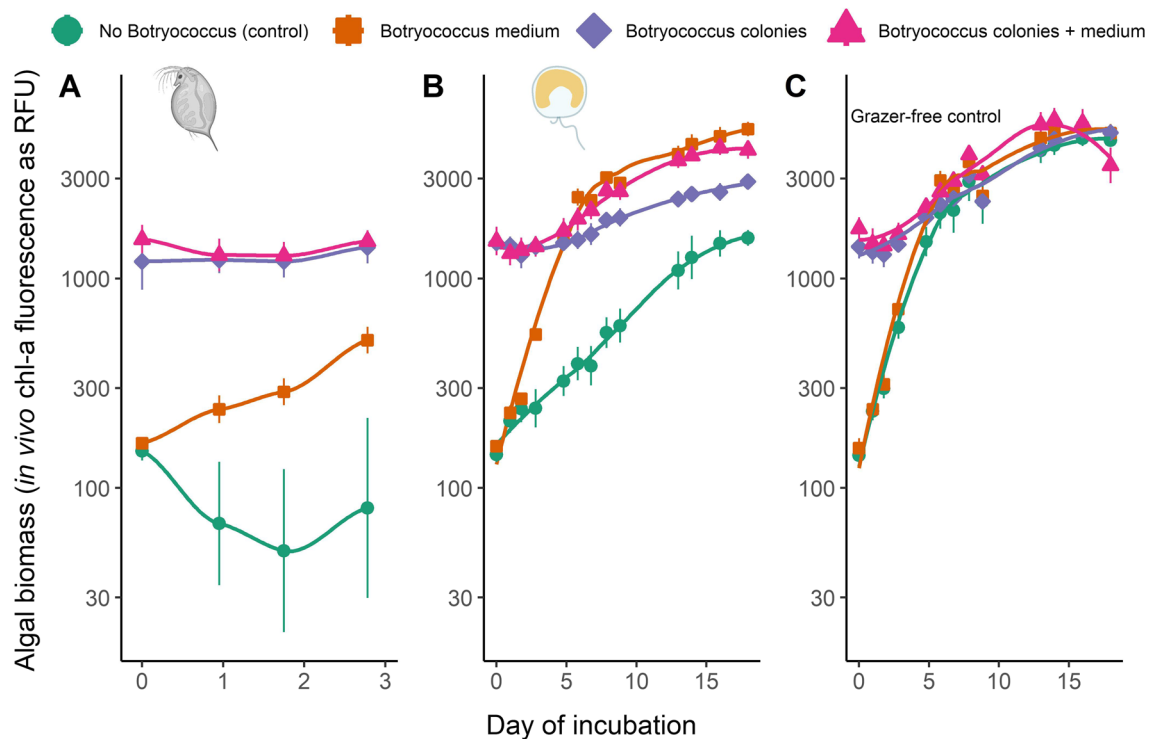


Fig. 1 Chlorophyll-*a* fluorescence (as a proxy for total algal biomass, log-scale) over time in the four *Botryococcus braunii* treatments, with (A) *D. magna* as grazer, (B) *Poterioochromonas malhamensis* as grazer, and (C) no grazer added. Both grazers caused strong reductions in biomass (circles in A, B) compared to the grazer-free controls (C) when *Nannochloropsis limnetica* was grown alone in fresh medium; however, loss of total algal biomass was mitigated with the addition of *B. braunii*'s filtered culture medium, *B. braunii* colonies

alone, and the combination of *B. braunii* medium and colonies. Points show mean and SD of replicates ($n=6$); lines represent loess fits. Note that fluorescence captures biomass of all photosynthetic organisms when present in a given treatment: *N. limnetica* (present in all treatments), *B. braunii* (present in half of treatments) and, to a lesser degree, the mixotrophic flagellate *P. malhamensis* (only in treatments shown in Fig. 1B)

Conversely, the presence of *B. braunii* filtrate inhibited grazing by *D. magna*, allowing *N. limnetica* density to increase despite the presence of the grazers, though max. chl-*a* fluorescence was still significantly reduced (mean 29% loss compared to grazer-free controls). The treatments applying *B. braunii* colonies and colonies + medium provided even further protection from *D. magna* grazing, with total algal biomass (i.e., *B. braunii* and *N. limnetica* combined) losses of only 4.6% and 10.1%, respectively, compared to grazer-free controls). The 95% CIs overlapping zero indicate no significant declines in biomass (Fig. 2A) or growth rate (Fig. 2C) when *B. braunii* colonies are present. ANOVA revealed significant individual effects of adding *B. braunii* medium as well as *B. braunii* colonies. Moreover, there was a significant interactive effect between the two, in terms of both algae biomass loss ($p < 0.02$) and growth rate change ($p < 0.001$); i.e., the effect of adding *B. braunii* medium is reduced when *B. braunii* colonies are present, and vice versa (see Table S1 for ANOVA tables). At the final time point, *N. limnetica* cell densities were highly variable across replicates, but on average were relatively low in the *B. braunii* medium

treatment ($7.8 \times 10^5 \pm 4.5 \times 10^5$ SD cells mL^{-1}). When *B. braunii* cells and medium were present, *N. limnetica* densities were $2.0 \times 10^6 \pm 2.1 \times 10^6$ cells mL^{-1} (Fig. 3); these cell densities are on average 46% lower than in the corresponding grazer-free control ($3.8 \times 10^6 \pm 1.0 \times 10^6$ cells mL^{-1}). While this indicates *N. limnetica* were still grazed upon, it also confirms that *B. braunii* prevents the rapid depletion of *N. limnetica* biomass that we observed in the absence of *B. braunii* (see control in Fig. 1A), and that the remaining biomass is comprised of a mixture of *N. limnetica* and *B. braunii*.

***Botryococcus braunii* inhibits grazing by *Poterioochromonas malhamensis* through both chemical and physical interference**

In the control treatment with no *B. braunii* present, *P. malhamensis* significantly reduced *N. limnetica* biomass (mean 67% decrease in max. chl-*a* fluorescence, Fig. 1B, 2B) and growth rates (Fig. 2D) compared to the grazer-free controls. However, the presence of *B. braunii* medium mitigated grazing losses by *P. malhamensis*, as max. chl-*a* fluorescence of

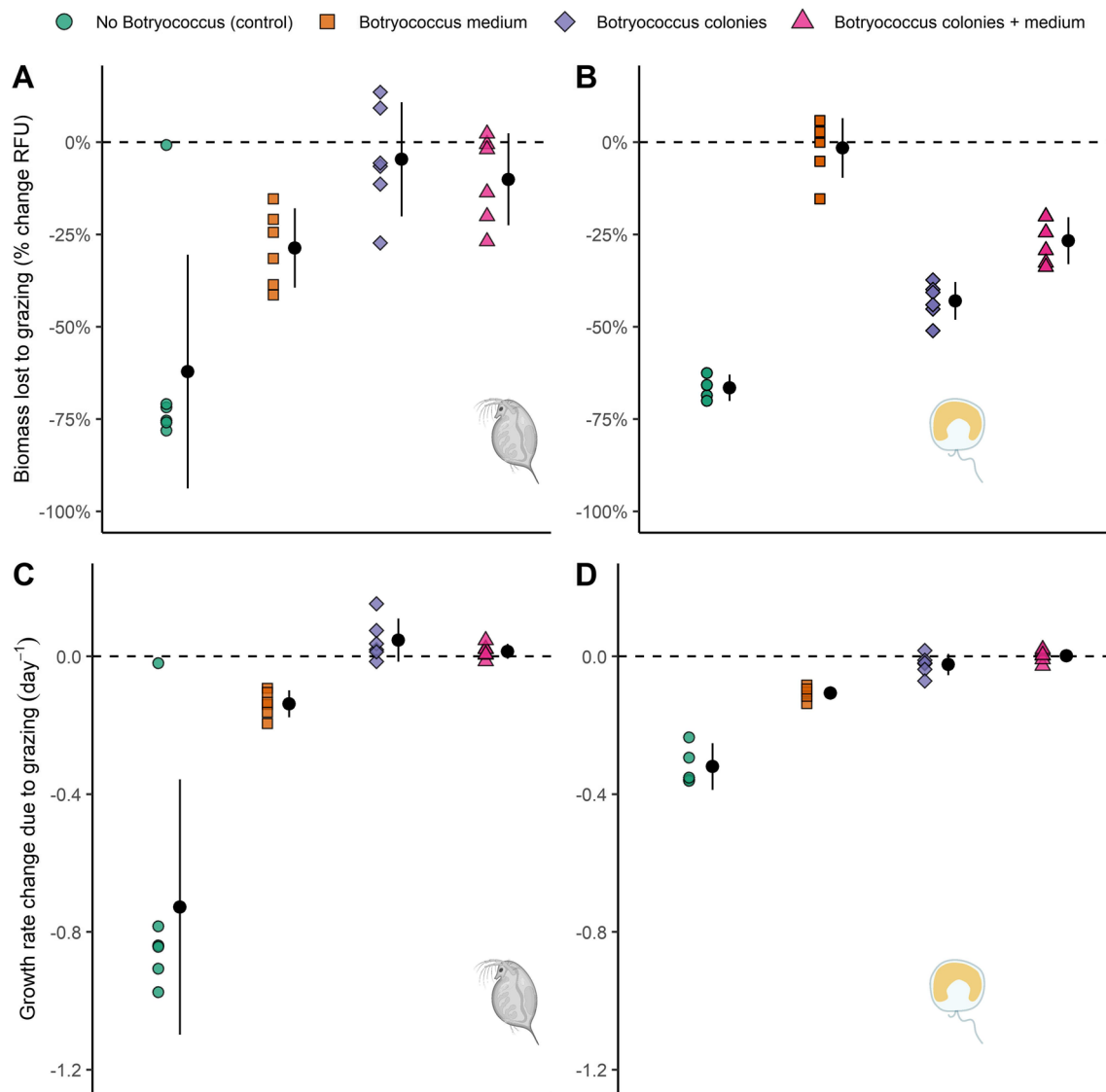


Fig. 2 Net effects of grazing on biomass and growth rate under different *B. braunii* treatments. Shown are grazing effects on (A, B) maximum chl-*a* fluorescence (as a proxy for total algal biomass) and (C, D) initial growth rates from day 0–3 caused by the different grazers (*D. magna* in A, C; *P. malhamensis* in B, D). Biomass loss calculations show the % change in maximum fluorescence (i.e., high-

est fluorescence value over time) in each experimental unit relative to the mean grazer-free control in the corresponding *B. braunii* treatment; growth rate changes show the difference in growth rate in each experimental unit compared to the mean grazer-free control. Points are data for individual replicates; black bars show mean and 95% CI per treatment

N. limnetica only decreased by a mean of 1.6% compared to grazer-free control (i.e., no significant biomass loss). In contrast to treatments with *D. magna* as grazer, the *B. braunii* colonies (43% decrease) and colonies + medium (27% decrease) treatments allowed for greater grazing losses to *P. malhamensis* than *B. braunii* medium alone over the full 18-day incubation (Fig. 2B). However, their initial (day 0–3) growth rates did not differ from those of the grazer-free controls (Fig. 2D). ANOVA again showed a significant interaction between effects of *B. braunii* medium and *B. braunii* colonies ($p < 10^{-5}$ for both biomass and growth rate; Table S1).

Final cell densities of *N. limnetica* were higher in *B. braunii* medium ($5.0 \times 10^6 \pm 2.4 \times 10^6$ cells mL⁻¹) when compared to colonies + medium ($2.7 \times 10^6 \pm 1.0 \times 10^6$ cells mL⁻¹), colonies only ($1.0 \times 10^6 \pm 5.0 \times 10^5$ cells mL⁻¹), or the treatment without *B. braunii* ($1.3 \times 10^6 \pm 4.0 \times 10^5$ cells mL⁻¹) (Fig. 3). Final counts of *P. malhamensis* showed that *B. braunii* medium reduced the density of *P. malhamensis* by 56% compared to the control, while treatments containing *B. braunii* colonies tended to cause increases in *P. malhamensis* cell density (Fig. 4A). *P. malhamensis* final density, however, was not correlated with *N. limnetica* final density (Fig. 4B; Spearman $\rho = -0.3$, $p = 0.4$).

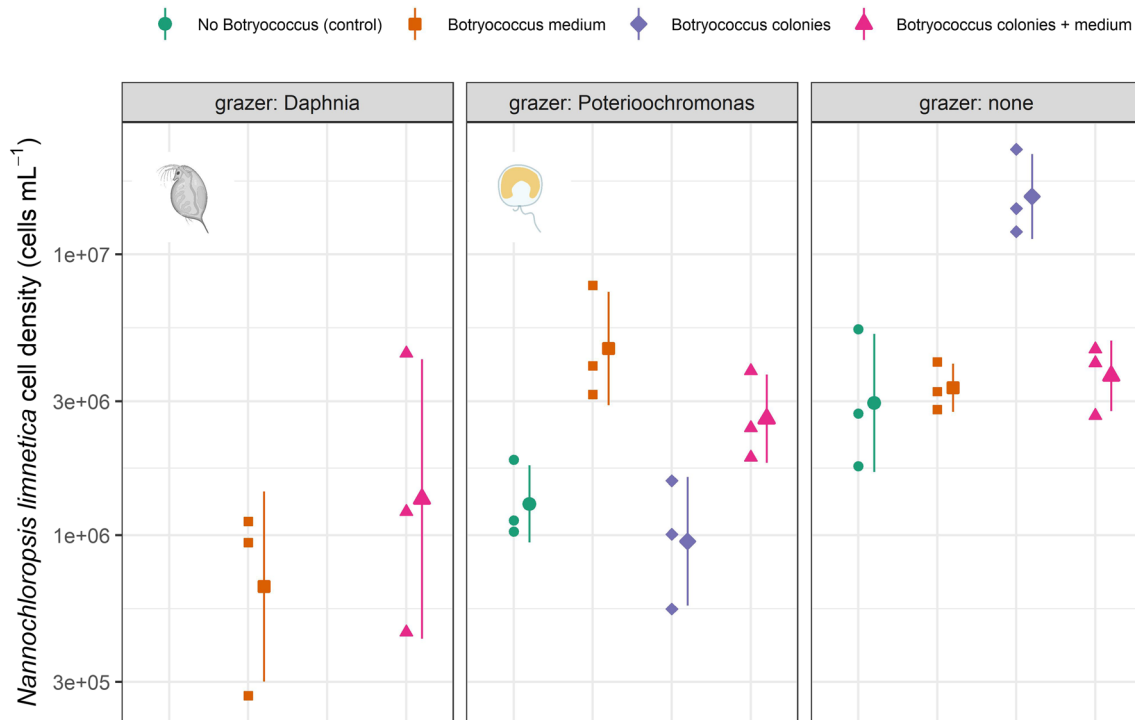
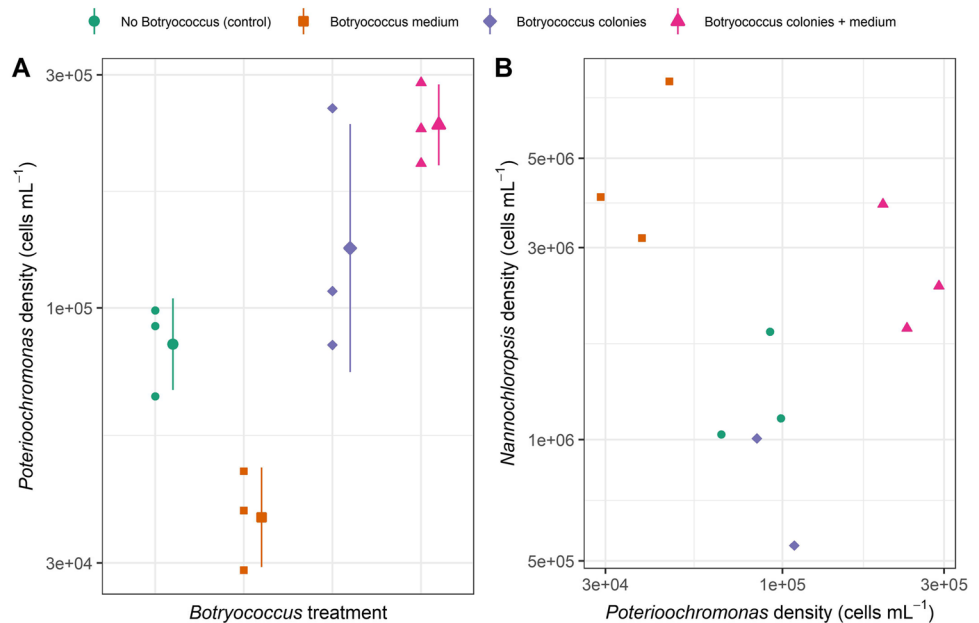


Fig. 3 Cell densities of *N. limnetica* at the end of the experiment (day 18). Data show counts for individual replicates alongside mean and SD of cell densities. Data are only shown for experimental units with

live *Daphnia*; missing *Daphnia* treatments are those in which *Daphnia* died prior to day 18. Cell counts were performed on a randomly chosen subset of 3 of 6 replicates

Fig. 4 **A** Cell densities of *P. malhamensis* at the end of the experiment (day 18). Data show counts for individual replicates alongside mean and SD of cell densities. Cell counts were performed on a randomly chosen subset of 3 of 6 replicates. **(B)** *P. malhamensis* densities plotted against *N. limnetica* densities. Each point represents cell densities in one experimental unit

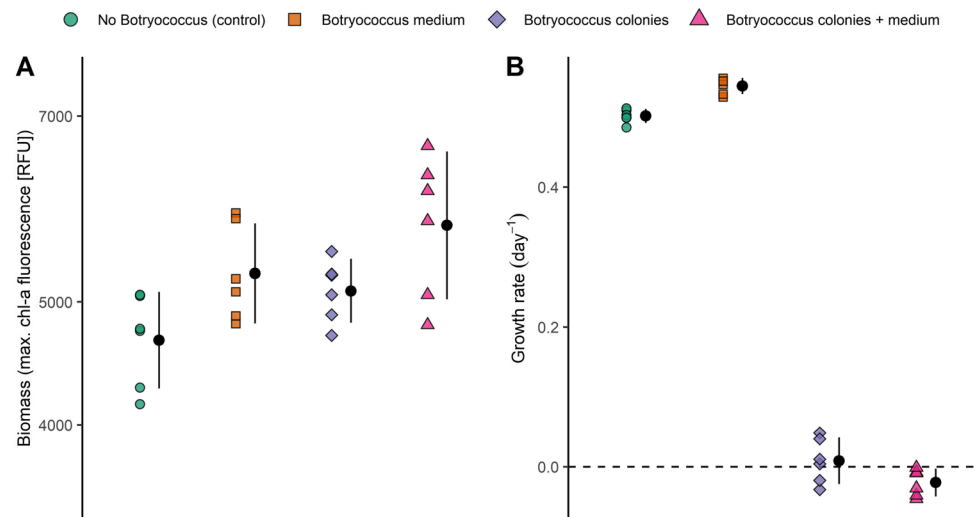


Botryococcus braunii* medium enhances growth of *Nannochloropsis limnetica

Compared to *N. limnetica* grown alone in fresh medium (max. fluorescence of $4678 \text{ RFU} \pm 384 \text{ SD}$ and growth rate of $0.50 \pm 0.01 \text{ day}^{-1}$), *N. limnetica* grown in the used

medium of *B. braunii* had 13% higher max. fluorescence ($5279 \text{ RFU} \pm 461 \text{ SD}$, $p=0.13$, Tukey HSD) and 8% higher growth rates ($0.54 \pm 0.01 \text{ day}^{-1}$, $p=0.007$, Tukey HSD), indicating an overall enhancement of growth due to *B. braunii* medium (Fig. 1C, Fig. 5). Max. fluorescence was highest in the treatment containing *B. braunii* colonies and medium

Fig. 5 Biomass in terms of maximum chl-*a* fluorescence (A) and initial day 0–3 growth rates (B) for different *B. braunii* treatments when no grazer is present. Points are data for individual replicates; black bars show mean and 95% CI per treatment



(5780 ± 718 SD, 24% higher than *N. limnetica* grown alone). ANOVA showed a significant interaction between effects of *B. braunii* medium and *B. braunii* colonies for growth rate ($p < 0.001$) and significant independent effects of *B. braunii* medium and *B. braunii* colonies for max. fluorescence ($p < 0.03$, Table S1). Growth rates were slower when *N. limnetica* was added to stationary phase *B. braunii*; however, the fact that biomass increased at all over time indicates the potential for synergistic interactions and overyielding in co-culture compared to monoculture. Moreover, final cell counts show that *N. limnetica* cell densities in the presence of *B. braunii* colonies and/or medium are at least as high as those when *N. limnetica* is grown alone (Fig. 3). In summary, *B. braunii* does not inhibit, and may even facilitate *N. limnetica* growth.

Discussion

The results of our study provide support for our hypotheses that (H1) *B. braunii* inhibits both grazers via a combination of chemical and physical factors, and (H2) *B. braunii* does not chemically inhibit *Nannochloropsis* growth. Our results, although they are limited to the laboratory microcosm scale, provide a precedent for engineering algal communities with multiple synergies. Specifically, we propose that *B. braunii* could simultaneously function as a crop protection agent for fast-growing algae such as *Nannochloropsis* species that can be harvested for higher-value products, while also producing extracellular hydrocarbons (Ennaceri et al. 2023) and exopolysaccharides (Wang et al. 2022).

Potential generalities, and context-dependencies, of using defended algae strains for crop protection

While we use *Nannochloropsis* as a focal ‘fast-grower’ we imagine that this principle could also apply to other small undefended taxa, e.g., *Synechocystis* that can be used for sustainable PHA bioplastic production (Koch et al. 2020), *Haematococcus* for antioxidants (Shah et al. 2016), or a variety of other small strains of interest for feed or fuel. Although in this study we use freshwater organisms, the general principle should apply equally well to brackish or saltwater cultivation. The fact that biomass loss was not completely prevented by *B. braunii* also suggests that this approach should be used in conjunction with complementary approaches to modify the biology and chemistry of pond systems for crop protection to more comprehensively manage complex pond communities (McBride et al. 2014; Day et al. 2017; Ma et al. 2017; Thomas et al. 2017; Fisher et al. 2019; Van Ginkel et al. 2020; McGowen et al. 2023).

Our results provide additional evidence that species-specific chemical inhibition can be used to inhibit grazers without inhibiting algal productivity. A previous meta-analysis showed the chemical compounds in recycled cultivation medium on average cause inhibitory effects on algal productivity (Loftus and Johnson 2017); therefore, finding algae species that are chemically compatible (as in our study) is of utmost importance, both for the purpose of recycling culture water and for the purpose of creating a selective chemical environment that favors low grazer densities. There is also potential to take advantage of dissolved metabolites produced by algae and bacteria that stimulate algal productivity,

for example plant growth hormones like indole acetic acid (Brisson et al. 2021; Negi et al. 2024). Indeed, our results suggest a positive effect of used *B. braunii* medium on *N. limnetica*, which warrants further study. *Nannochloropsis gaditana* growth, for example, increases when grown mixotrophically with added glucose and glycerol (Menegol et al. 2019); therefore, a possible explanation is that in our study *N. limnetica* metabolized exopolysaccharides excreted by *B. braunii*, thus enhancing its growth. However, an increase in dissolved organic carbon from *B. braunii* exudates could also cause indirect effects, such as enhancing the growth of bacteria or even mixotrophs like *P. malhamensis*. We did in fact note higher *P. malhamensis* density when *B. braunii* colonies and medium were present (Fig. 4A), suggesting the possibility that these mixotrophs benefited from more exudates and/or free-living bacteria in such *B. braunii* treatments. However, we observed no clear effects of *P. malhamensis* density on *N. limnetica* cell density (Fig. 4B); it is thus possible that *P. malhamensis* switched to smaller bacterial prey or osmotrophy instead of ingesting *N. limnetica*. The influence exerted by *B. braunii*'s diverse microbiome (Blifernez-Klassen et al. 2021) on grazers and pathogens in algal systems also warrants in-depth investigation. In summary, the net effect of *B. braunii* in our experiment is likely a combination of inhibition and stimulation of our study organisms, both direct and indirect. Future research, especially over longer time periods and at larger scales, is needed to understand the complex dynamics that may arise even from a relatively simple system such this.

Although this microcosm study provides a clear proof of concept for a community engineering strategy using *B. braunii* to inhibit grazers and stabilize culture biomass, we emphasize that many factors will determine whether it is as effective at commercial scale as in our lab study. For example, we assess only one genotype of each organism; other distinct genotypes of each organism studied here are likely to have differences in key traits (e.g., chemical exudate profiles or colony size across strains/clades of *B. braunii*, differential tolerance to allelopathic inhibition in target algae such as *Nannochloropsis*), which could either enhance or diminish the net protective effect of *B. braunii*. While free fatty acid excretion has been linked to chemical inhibition by *B. braunii* (Chiang et al. 2004; Thomas et al. 2023), there is likely much more to uncover regarding the mechanisms of inhibition caused by the complex suite of bioactive metabolites excreted by different *B. braunii* strains. Different species or genotypes of grazers (or combinations of grazers), and other types of pests like viral, fungal, or bacterial pathogens may (or may not) be inhibited in a similar manner as the two grazers used here. It should also

be noted that our *B. braunii* medium treatments represented a single pulse disturbance in terms of chemical inhibition and thus could have temporary effects; therefore, impacts of chemical inhibition may differ if applied in a continuous or semi-continuous manner. While we used high densities of *B. braunii*, reflecting its maximum biomass at carrying capacity, using a lower relative density of *B. braunii* could alter results by changing the total strength of interactions with both *Nannochloropsis* and with grazers. Additionally, effects of other abiotic factors (e.g., temperature, salinity, pH/alkalinity, CO₂ addition), which are outside the scope of this study, could significantly alter the effects of chemical inhibition and thus be optimized in tandem with the crop protection strategy described in our study.

Future perspectives on the microalgae “eco-refinery” concept

The biorefinery concept describes an approach in which commercial facilities process a variety of cellular components, usually from an algal monoculture, into distinct bio-based products in order to maximize the economic and environmental benefits of algae farming (Laurens et al. 2017; Khoo et al. 2019). Complementary to the biorefinery approach, the “eco-refinery” framework described in this study additionally allows the separation of different algae species, grown together in the pond ecosystem, into different fractions prior to downstream conversion pathways. Because the size of *B. braunii* colonies (up to 200 µm in diameter) is significantly greater than cell size of strains of industrial interest (e.g., *Nannochloropsis* or *Picochlorum*, which are < 5 µm), it is feasible to selectively harvest the large *B. braunii* colonies (e.g., with a 20-µm filtration apparatus), perform non-destructive extraction of hydrocarbons in the extracellular matrix (Moheimani et al. 2013; Griehl et al. 2015), and return live colonies to ponds. The under 20-µm fraction, containing primarily the target small algae, could then be dewatered and processed using current biorefinery methods. The relative cell densities of each species in a pond could also be controlled by dynamically changing the fraction of each that is harvested and removed from/returned to the system. It is the opinion of the authors, therefore, that the eco-refinery approach has the potential improve bioprocess design while also preventing pond crashes due to pests. We again stress, however, that these methods must be tested and validated at larger scales. We therefore suggest that an expanding branch of research should evaluate potential benefits of integrating ecologically-based crop protection measures with novel bioprocess engineering pathways to enhance the reliability and efficiency of algal bioproduct generation.

Conclusions

Our study provides a proof of concept that *B. braunii*, an alga that excretes extracellular hydrocarbons and polysaccharides, can also protect small, fast-growing algae from grazing pressure, thus providing early evidence for a novel mechanism of protecting algal crop productivity in designed polycultures at mass scale for a suite of complementary renewable bioproducts. Future work is needed to test and validate this novel strategy at pilot and commercial scales.

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Data availability All data and code used are publicly available on Zenodo (<https://doi.org/10.5281/zenodo.13237033>).

Declarations

Competing interest The authors declare no competing interests.

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