Impact of Brewer's Spent Grain on Freshwater and Marine Harmful Algal Bloom Species

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Abstract

Barley straw bales are commonly used to control cyanobacterial growth in lakes, but the method is time-consuming, labor-intensive, and its effectiveness requires deployment of bales prior to bloom formation. This study investigates brewer's spent grain (BSG), a byproduct of the brewing process, as an alternative source of allelopathic chemicals shown to negatively impact toxic microalgae. Water extracts of BSG, barley straw, and *Sargassum natans* were tested for their ability to inhibit the growth of toxin-producing (*Microcystis aeruginosa* and *Karenia brevis*) as well as non-toxin-producing (*Tetradesmus obliquus* and *Phaeodactylum tricornutum*) algal species. BSG extracts at concentrations above 250mg/L effectively inhibited the growth of both freshwater and marine toxin-producing species (*M. aeruginosa* and *K. brevis*), while exhibiting no significant effect on the diatom and chlorophyte species tested (*T. obliquus* and *P. tricornutum*). Additional experiments using antibiotics with *K. brevis* suggest that growth inhibition may be mediated by changes in the bacterial community, though the specific mechanism of *M. aeruginosa* death remains unresolved. A microcosm experiment further evaluated the impact of BSG extract on a natural bloom of cyanobacteria (*Raphidiopsis raciborskii*) in lake water. Application of 250 mg/L BSG extract to natural lake water shifted the community composition from cyanobacteria to chlorophyte dominance. These findings highlight the potential use for a brewery's waste product as a cost-effective tool for managing harmful algal blooms. However, the high concentrations required, excess nutrient content in BSG, and impact on bacterial communities indicate limitations for large-scale application.

Keywords: brewer's spent grain, HABs, allelochemicals, Bloom Control, cyanobacteria

1. Introduction

Harmful algal blooms (HABs) are a global concern in both freshwater and coastal systems, posing serious threats to public health, water resources, and local economies (Anderson et al., 2008; Hoagland and Scatasta, 2006; Wells et al., 2019). As a result, scientists and managers are focusing more on HAB prediction, prevention, and control. The current chemical control methods include algicides such as copper sulphate, chlorine, and hydrogen peroxide (Gibson, 1972; Lusty and Gobler, 2020; McKnight et al., 1983; Rodríguez et al., 2007). However, these chemicals are often impractical for large scale use as the solutions can produce undesired by-products and/or impact non-targeted organisms (Anantapantula et al., 2025; Lusty and Gobler, 2020). Consequently, there is a growing need for alternatives to synthetic chemical algicides that are more species specific and safe for use.

The use of natural allelochemicals produced by plants and bacteria has received considerable attention as alternatives to synthetic algicides. Depending on the plant, the source of allelochemicals can include phenolic acids, flavonoids, tannins, or fatty acids (Gao et al., 2020; Lv et al., 2021; Mecina et al., 2019, 2017; Xie et al., 2023). Plant allelochemicals often have negligible side effects, can be highly species selective, and capable of degrading in aquatic environments over time (Sun et al., 2019; Zhu et al., 2021). These characteristics make them suitable candidates for safe and sustainable HAB control.

A variety of plants including aquatic macroalgae and terrestrial species have been studied for their effectiveness at suppressing toxin-producing microalgae (Sylvers and Gobler, 2025; Tang and Gobler, 2011; Wang et al., 2007; Zerrifi et al., 2018; Zhao et al., 2019). One of the most well studied and commonly used methods to control cyanobacterial blooms is the deployment of barley straw bales, which has been used in lakes since the 1970s (Barrett et al., 1999; Gibson et al., 1990; Iredale et al., 2012; Sellner et al., 2014; Waybright et al., 2009).

However, this method is time consuming, labor intensive, and its effectiveness is dependent on deploying bales approximately 12 weeks prior to the bloom formation. As a result, barley straw is more suitable as a preventative measure than a reactive control strategy.

Scientists have expanded the research for cyanobacteria specific algicidal compounds to include plant waste products, including pomegranate peels (Chen et al., 2019), watermelon rinds (Zhang et al., 2020), and rice and wheat husks (Ma et al., 2018). One potentially viable control solution that has yet to be studied is brewer's spent grain (BSG), the insoluble fraction of barley husks that constitute up to 85% of breweries total by-products. Currently, BSG is primarily discarded or repurposed as animal feed. However, with rising disposal costs, both breweries and scientists are exploring alternative applications for this antioxidant-rich resource (Allegretti et al., 2022; Lynch et al., 2016). Around 36.4 million tonnes of BSG is produced annually worldwide and so it is readily available at low or no cost (Nyhan et al., 2023). Since the husks contain much of the barley's phenolic components, the phenolic acids and flavonoids are an estimated fivefold concentration higher in BSG compared to that of barley straw (Stefanello et al., 2018). The brewing process heats and mashes the barley husks, which begins the breakdown of the plant material. Consequently, BSG is already partially decomposed and may exert algicidal effects more quickly than barley straw, which relies on microbial degradation over time. These characteristics suggest that BSG could serve as an economical and more immediately effective source of natural algicides.

The primary objective of this study was to assess the potential of BSG to inhibit the growth of harmful algal bloom species in both freshwater and marine environments. Specifically, we report on (1) the growth-inhibiting effects of BSG extract compared to barley straw and *Sargassum natans*, on the cyanobacteria *Microcystis aeruginosa,* chlorophyte *Tetradesmus*

obliquus, dinoflagellate *Karenia brevis*, and diatom *Phaeodactylum tricornutum*; (2) whether any observed inhibition from BSG are due to chemical activity or bacteria overgrowth; and (3) the effect of BSG extract on freshwater phytoplankton community composition during a natural bloom of *Raphidiopsis raciborskii*.

2. Methods

2.1 Preparation of plant extracts

Brewer's spent grain (BSG) was obtained from Diamondback Brewing Company (Baltimore, MD) in July 2019, following the brewing of a pilsner with 100% barley husks as the grain source. *Sargassum natans* was collected ship-side using a handheld net in the Sargasso Sea in July 2016. Barley straw (*Hordeum volgare*) was collected from barley bales in July 2019. *Sargassum* and barley were rinsed with deionized (DI) water then *Sargassum*, barley, and BSG were dried at 70℃ for 48 hrs and ground into powder at 25,000 rpm. Fifteen grams of the powdered plants were then transferred to 250 mL autoclaved glass bottles with 200 mL ultrapure H_2O (75 g/L). The bottles were placed on a shaker table at 100 rpm speed at 20℃ for 24 hrs and then centrifuged at 2,000xg at 20℃ for 10 min. The resulting supernatant was filtered through 25mm GF/F filters followed by 0.2 µm polycarbonate filters into autoclaved glassware and kept at -20℃ until further use. To determine the organic matter concentrations, 3 mL aliquots from each final extract were dried. The resulting dry weights were 13.5 mg/mL for both BSG and *Sargassum* extracts, and 3 mg/mL for the barley straw extract. Algal culture treatments were dosed based on equal dry weights of organic matter across the different plant extracts.

2.2 Plant extract experiments

Microalgae were exposed to 50mg/L, 100mg/L, 250mg/L dry weight of BSG, *Sargassum*, and barley extracts (n=3), using 250 mL Erlenmeyer flasks (autoclaved at 121℃ for 20 min),

99 with 115 mL medium, 25 mL culture and respective amount of 10 mL plant extract: ultrapure
100 H_2O depending on the specific concentration. The controls had 10 mL ultrapure H_2O added with
101 no plant extract. At the same time, additional flasks of cultures were exposed to 6 mg/L purified
102 luteolin dissolved in dimethyl sulfoxide (DMSO) as a positive control (10 mg/mL stock solution)
103 in *Tetradesmus obliquus* and *Phaeodactylum tricornutum* experiments (n=3). Since DMSO is
104 known to impact microalgal growth, the luteolin treatment was compared to a DMSO control of
105 the same addition, 0.12% v/v (n = 3). The 50mg/L, 100mg/L, 250mg/L dry weights were equal
106 to extract doses of 0.2%, 0.4%, 1% of BSG and *Sargassum*, and 1%, 2%, and 5% barley.
107 *Microcystis aeruginosa* (strain LE3) and *T. obliquus* (strain HTB1) were cultured in BG-11
108 medium, 20°C, and 50 µmol photons $m^{-2}s^{-1}$ PAR with a 12:12 light cycle. Cultures in
109 exponential growth were inoculated at $1x10^6$ cells mL^{-1}. Cell counts were performed on days 0,
110 1, 2, 4, 6, and 8 during the experiment with *T. obliquus* and days 0, 1, 2, 4, and 6 during the
111 experiment with *M. aeruginosa*. Photosynthetic efficiency was measured on days 1 and 2. The
112 growth and percent mortality of *M. aeruginosa* were determined by counting 25 µL on medium
113 speed using a flow cytometer (BD Accuri™ C6), where events with fluorescence above 10^5
114 measured under FL4-H wavelength 640nm were considered alive. The growth and percent
115 mortality of *T. obliquus* were determined using a hemocytometer.
116 *P. tricornutum* (strain UTEX 646) was cultured in 20 ppt F/2 medium, 20°C, and 100 µmol
117 photons $m^{-2}s^{-1}$ PAR with a 12:12 light cycle. *P. tricornutum* in exponential growth was
118 inoculated at $5x10^5$ cells mL^{-1}. Cell counts were performed on days 0, 1, 2, 4, 6, and 8, using a
119 hemocytometer. Photosynthetic efficiency was measured on days 1 and 2.
120 *Karenia brevis* ("New Pass" strain, CCMP 2228) was cultured in 32 ppt modified F-Si
121 medium (f/2 medium of nutrients), 24°C, and 100 µmol photons $m^{-2}s^{-1}$ PAR with a 14:10 light

cycle. *K. brevis* in exponential growth was inoculated at 3,000 cells mL^{-1}. Bacteria counts and cell growth counts were performed on days 0, 1, 2, 4, 6, and 8. Bacteria counts were performed by adding 100 µL sample to 100 µL of 1 µM SYBR green I Nucleic Acid Gel stain, incubating in the dark for 10 min, and counting 25 µL on medium speed using flow cytometer (BD Accuri™ C6). Events with fluorescence above 10^4 measured under FL1-H wavelength 550nm were considered bacteria. Five mL samples were collected from each flask for cell counts and counted using a Multisizer 4e Coulter Counter equipped with a 70 µm aperture. pH measurements were taken on days 0, 4, 6, and 8.

2.3 Antibiotics experiment

Karenia brevis ("New Pass" strain, CCMP 2228) was cultured in modified F-Si medium (f/2 nutrients), 24°C, and 100 µmol photons $m^{-2}s^{-1}$ PAR with a 14:10 light cycle. *K. brevis* in exponential growth was inoculated at 500 cells/mL and exposed to either the control of 10 mL ultrapure H_2O, 0.12% DMSO, 6 mg/L purified luteolin dissolved in DMSO, 50 mg/L BSG extract, or 200 mg/L BSG extract, using 250 mL Erlenmeyer flasks (autoclaved at 121°C for 20 min), with total volume of 200 mL (n=6). Bacteria counts and cell growth counts were measured on days 0, 1, 2, 4, 6, and 8, using a flow cytometer and culture counter as described in Section 2.2. On day 4, 25 mL samples were collected from each flask, filtered onto a 0.2 µm polycarbonate filters, and stored at -20°C for subsequent bacterial community composition analysis.

2.4 Lake water experiment

Ten liters of water was collected from the surface of Williston Lake in Denton, Maryland during a bloom of *Raphidiopsis raciborskii* on Sept. 4, 2019 (38°49'29.9"N 75°50'19.4"W). The water was immediately filtered through 150 µm filter to remove large zooplankton and debris

145 before transportation. The filtered lake water was homogenized and 500 mL was transferred to
146 600 mL sterile culture flasks. The flasks were dosed with either five mL ultrapure H_2O (control),
147 five mL BSG extract (250mg/L; BSG 1%), or 0.5 mL BSG extract/4.5 mL ultrapure H_2O
148 (25mg/L; BSG 0.1%) (n=3). Flasks were placed in an incubator at 20°C, 12:12 light: dark cycle,
149 and 100 μmol photons $m^{-2}s^{-1}$ PAR.

150 The phytoplankton community composition was sampled at day 0, 2, 4, and 6 after thorough
151 mixing, taking 4 mL aliquots, and preserving using 1% Lugol's solution. The preserved
152 phytoplankton samples were analyzed by identifying plankton to the lowest possible taxa in x100
153 magnification until at least 300 algal cells were counted. Pigment samples were collected at day
154 0 and 6 by filtering 5 ml through 25 mm GF/F and freezing at -80°C. The pigment analysis was
155 performed via HPLC at Horn Point laboratory (Cambridge, MD). Water quality data including
156 pH, total alkalinity, NH_4^+, NO_2^-, PO_4^{3-}, and NO_3^- were analyzed by Zooquatics (Baltimore, MD)
157 in the inoculum, spent grain extract, and day 6 flasks.

158 *2.5* Heterotrophic bacterial community analysis

159 DNA barcoding analysis was performed to assess the changes in community composition of
160 heterotrophic bacteria in the *K. brevis* cultures with and without the use of antibiotics. Filters
161 were aseptically cut in half (using sterile scissors and tweezers) and each placed into a DNeasy
162 PowerLyzer Powersoil Kit PowerBead Tube (Qiagen, Valencia, CA, USA). From this point, the
163 kit protocol was followed, and the isolated DNA was eluted into 100uL of the C6 solution. DNA
164 from the variable V4 regions of the 16S rRNA gene was then amplified using the modified 515F
165 and 806R primers and sequenced via a MiSeq 16S amplicon run (Apprill et al., 2015; Parada et
166 al., 2016).

167 *2.6* Phenolic Content analysis

Total Phenolic Content (TPC) was estimated for the plant extracts using the Folin-Ciocalteu method of Singleton et al (1974), with some modifications. 100 µL of the plant extracts were added to 900 µL ultrapure H_2O and vortexed (n=3). 100 µl of 1 M NaOH was added, followed by 120 µl of Folin-Ciocalteu reagent, vortexed, and put in the dark for five minutes. 300 µl of 200g/L Na_2CO_3 was added, vortexed, and samples were put into the dark for one hour (Singleton et al., 1974). A 250 µl aliquot was transferred to a flat bottom 96 well plate and adsorption at 765 nm was measured on a SpectraMax i3x multi-mode microplate reader. The samples were compared to a gallic acid standard curve from 1 to 50 µg/mL and presented in gallic acid equivalents (GAEs).

The complex dissolved organic matter (DOM) with a special focus on the phenolic content was analyzed using a 12 Tesla Bruker Solarix Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) (Burker Daltonics GmbH, Bremen, Germany). To separate phenolic content and remove salts, 50 mL of each 0.2 µm filtered plant supernatant were acidified to pH 2 using concentrated HCl and solid phase extracted using Agilent Bond Elut PPL (1 g, 3mL) cartridges. This solid-phase extraction (SPE) technique has been previously described (Dittmar et al., 2008; Powers et al., 2019; Takeda et al., 2013). Cartridges were activated with 10 mL MeOH, rinsed with 20 mL pH 2 pure water, and 50 mL acidified plant supernatant was passed through. The cartridge was then washed with 20 mL 0.1% formic acid water and dried. Phenolic content was eluted with 10 mL MeOH into a 20mL acid-washed amber glass vial with a Teflon-lined silicon lid and 5 mL of the SPE extracted sample was dried via speedvac system without the use of light and stored at -20°C until analysis. The 5 mL dried SPE extracted plant samples were redissolved in 1 mL MeOH prior to injection. The samples were directly infused at 3 µL min^{-1} flow rate and 500 scans were averaged. The mass error was always better than 0.2 ppm,

which allowed very accurate molecular formula assignments of the CHO signatures. A previously established network approach was used to assign molecular formulas (Tziotis et al., 2011). Only molecules that contain CHO equal or below H/C ratios of 1 were plotted in van Krevelen space to compare molecular profiles of the highly aromatic compounds, which contain the polyphenolic signatures.

2.7 Photosynthetic efficiency analysis

Photosynthetic efficiency were measured at 24hrs and 48hrs using a pulse amplitude modulated spectrofluorometer (PAM-2500, WALZ, Germany), integrated with a computer with data acquisition software (PamWin 3.2) using the methods of Srivastava et al 2021 (Srivastava et al., 2021). 1 x 10^7 cells from each flask were centrifuged at 2,000xg 10min at 20°C, resuspended in 400 µl of culture media and transferred to a KS-2500 suspension cuvette (WALZ, Germany). After 15 min of dark acclimation, the minimal fluorescence yield (F_0) was determined with <1 µmol $m^{-2}s^{-1}$. The maximum fluorescence yield (F_m) was measured after a 0.3s pulse of a saturating light (630 nm, intensity 3000 µmol photons $m^{-2}s^{-1}$). The ratio of F_v/F_m was used to determine the maximal PSII photochemical efficiency, where F_v is the difference between the maximum (F_m) and initial (F_0) fluorescence after dark acclimation. Only *M. aeruginosa*, *P. tricornutum*, and *T. obliquus*, were analyzed for photosynthetic efficiency due to the high cell volumes needed for analysis and the difficulty in concentrating *K. brevis* cells.

2.8 Statistics

Results are expressed as mean values ± standard deviation of the mean. Significant differences among mean response of microalgae and bacteria during exposure of extracts was determined by one-way analysis of variance (ANOVA). An ANOVA was also performed to test differences in total cells/mL and nutrients. Where significant differences were detected, the

Tukey's HSD post hoc test was used for separation of means. Data were assessed for normality (Shapiro-Wilk test) and for homogeneity of variance (Levene test) at the 0.05 significance level. When non-normality or non-homogeneity of variance was found, the data was logarithmically transformed and if tests still failed, non-parametric Kruskal-Wallis test was conducted followed by a Dunn's test. Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis similarity matrix was performed to visualize differences in phytoplankton and bacteria composition between treatments. A permutational multivariate ANOVA (PERMANOVA) was performed to compare the phytoplankton community composition between groups when lake water was exposed to BSG and between bacterial communities when *K. brevis* was exposed to concentrations of BSG with and without antibiotics. This analysis was performed using 'vegan' package in R. Statistical analyses were carried out with R studio (Version 4.2.2 R script). Algal growth responses were expressed as percentages of that of the control.

3. Results

3.1 Exposure of microalgae to brewer's spent grain, *Sargassum*, and barley extract

Plant extract doses were normalized by adding equal amounts of dry organic matter to the culture flasks, resulting in final concentrations of 50, 100, and 250 mg/L. However, during the 24-hour aqueous extraction process, greater amounts of material dissolved from the BSG and *Sargassum* (13.5 mg/mL dry weight) compared to the barley straw (3 mg/mL dry weight). As a result, five times more barley straw extract (1, 2, and 5% v/v) was required to match the target dry weight concentrations, compared to BSG and *Sargassum* extracts (0.2, 0.4, and 1% v/v). Despite normalization by dry organic matter, the total phenolic content (TPC) differed between extracts. In BSG treatments, final TPC concentrations were 0.53, 1.07, and 2.67 mg/L GAE in 50, 100, and 250 mg/L extract treatments (178 µg/mL GAE eq in initial BSG aqueous extract).

In *Sargassum* treatments, final TPC concentrations were 0.49, 0.99, and 2.48 mg/L GAE in 50, 100, and 250 mg/L extract treatments (165 µg/mL GAE eq in initial *Sargassum* aqueous extract). In contrast, barley straw treatments yielded significantly higher TPC concentrations: 3.22, 6.43, and 16.08 mg/L GAE in 50, 100, and 250 mg/L extract treatments (229 µg/mL GAE eq in initial Barley aqueous extract).

FT-ICR-MS spectra of the three plant extracts exemplified the diversity and differences in detected signals in the aromatic compound space (CHO, <1 H/C), which includes phenolic content (Fig. 1). BSG has reduced and low oxygen content signatures, not found in Barley or *Sargassum*. Alternatively, Sargassum has a group of reduced and highly oxidized polyphenols in their chemical space (low H/C, high O/C ratio).

No growth inhibition or enhancement was observed in diatom *P. tricornutum* or chlorophyte *T. obliquus* at any concentration of the three plant extracts tested or luteolin (Fig. S1&S2). Similarly, the mean photosynthetic efficiency of photosystem II (F_v/F_m) in both species at 24 hrs was not significantly different from the controls. However, after 48 hrs, *T. obliquus* exhibited significantly higher F_v/F_m values in cultures treated with 50mg/L BSG (0.57±0.004) and 100 mg/L barley (0.54±0.01) compared to the control (0.44±0.04) (ANOVA post-hoc Tukey; $F=13.47$, $p<0.001$; Fig. 2A). After 48 hrs, *P. tricornutum* exhibited significantly higher F_v/F_m values in cultures treated with 250mg/L BSG (0.4±0.002), 100 mg/L barley (0.4±0.01), 250 mg/L barley (0.39±0.02), and 250 mg/L *Sargassum* (0.37±0.03) compared to the control (0.24±0.05) (ANOVA post-hoc Tukey; $F=6.03$ $p<0.001$; Fig. 2B).

Both BSG and barley extract reduced *M. aeruginosa* growth at concentrations ≥100 mg/L after two days of exposure, exhibiting typical concentration-dependent response curves (Fig. 3). By day six, cell densities were reduced by 61% and 89% in cultures treated with 100mg/L and

250 mg/L BSG extract, and by 33% and 89% with 100mg/L and 250mg/L barley extract. No growth inhibition was observed in cultures treated with *Sargassum* extract at any concentrations tested. Photosynthetic efficiency (F_v/F_m) in *M. aeruginosa* at 24 hours was significantly lower in cultures exposed to 100 mg/L barley (0.15±0.03), 250mg/L barley (0.10±0.05), and 250 mg/L BSG (0.12±0.03), compared to the controls (0.32±0.12) (ANOVA post-hoc Tukey; $F=12.54$ $p<0.001$). At 48 hrs, mean F_v/F_m was significantly higher in cultures treated with 50mg/L BSG (0.38±0.01), 50mg/L barley (0.36±0.02), and 100 mg/L *Sargassum* (0.33±0.03), while significantly lower values were observed in cultures treated with 100mg/L (0.15±0.009) and 250mg/L (0.02±0.02) BSG, 250mg/L barley (0.14±0.012), and 250mg/L *Sargassum* (0.15±0.05), compared to the control (0.23±0.03) (ANOVA post-hoc Tukey; $F=60.3$ $p<0.001$; Fig. 2C).

A noticeable decline in *K. brevis* cell concentrations was observed by day two in all 250mg/L plant extract treatments (Fig. 3). By day six, cell densities were reduced by 90% and 98% following treatment with 100mg/L and 250 mg/L BSG extract, by 99.7% following treatment with 250mg/L barley extract, and by 99.4% following treatment with 250mg/L *Sargassum* extract. No significant growth inhibition was observed at lower concentrations of any of the plant extracts. Compared to control, bacterial abundance, increased significantly within 24 hrs of exposure to all plant extract treatments at all concentrations, and remained significantly higher during the experiment (ANOVA post-hoc Tukey; $p<0.001$).

3.2 Exposure of *Karenia brevis* to brewer's spent grain with and without antibiotics

Growth patterns of *K. brevis* following exposure to varying concentrations of BSG extracts with and without antibiotics are presented in Fig. 4A. The addition of antibiotics did not significantly impact the growth of *K. brevis* in control cultures over the 8-day experiment (paired t-test; $t=-1.42$ $p=0.17$). However, in control cultures, antibiotics significantly reduced bacterial

283 abundance through day 6 (ANOVA; $F=34.36$ $p<0.01$[day 1], $F=132.7$, $p<0.001$[day 2]; $F=25.84$
284 $p<0.01$[day 4], $F=38.58$ $p<0.01$[day 6], $F=6.22$ $p=0.067$[day 8]). In BSG extract treatments,
285 bacterial counts were also significantly reduced in cultures with antibiotics compares to those
286 without antibiotics (Fig. 5). This effect persisted for two days in 50 mg/L treatments (ANOVA;
287 $F=643.5$ $p<0.001$[day 1], $F=478.7$ $p<0.001$[day 2]) and for four days in the 200mg/L treatments
288 (ANOVA; $F=379.7$ $p<0.001$[day 1], $F=124.7$ $p<0.001$[day 2], $F=8.208$ $p<0.05$[day 4]).
289 *K. brevis* cell densities treated with 6 mg/L luteolin were reduced by 90% by day six,
290 with similar reductions observed in flasks with and without antibiotics (Fig. 4B). Treatment with
291 200 mg/L BSG extract without antibiotics resulted in a 93% cell reduction compared to the
292 control flasks by day six. In contrast, 200 mg/L BSG extract with antibiotics did not affect
293 growth through day four, but cell densities were reduced by 70% by day eight. The 50 mg/L
294 BSG extract with antibiotics did not significantly affect growth throughout the experiment, while
295 50 mg/L BSG extract without antibiotics produced variable outcomes, as indicated by the large
296 standard deviations in cell densities, despite similar bacterial abundances across replicates (Fig. 4
297 and 5).
298 Bacterial community composition at the genus level differed significantly between
299 treatments and antibiotic use (PERMANOVA; Treatment: $R^2= 0.26$ $F=3.43$ $p<0.001$,
300 Antibiotics: $R^2= 0.12$ $F=6.63$ $p<0.001$, Treatment*Antibiotics: $R^2= 0.27$ $F=3.56$ $p<0.001$; Fig. 6).
301 Shannon Species Diversity was significantly lower in flasks exposed to 50mg/L BSG extract
302 without antibiotics compared to those with antibiotics (ANOVA; $F=5.11$ $p<0.001$). The BSG
303 50mg/L and 200mg/L treatments without antibiotics were dominated by *Alteromonas spp.* (81.65
304 ± 10.53% compared to 7.89 ± 4.57% with antibiotics). With antibiotics, the community had
305 greater diversity, with increased relative abundance of *Blastopirellula spp.* (from 0.01% to 55.71

306 ± 22.56%) and *Bacillus spp.* (from 0.07 ± 0.07% to 6.57 ± 2.5%) (Fig. 6). In the control, DMSO,
307 and 6mg/L luteolin treatments, the relative abundance of *Marivita* sp. declined with antibiotic
308 addition (from 49.76 ± 6.4% to 16.6 ± 11.77%) (Fig. 6).
309 3.3 Exposure of *Raphidiopsis raciborskii* bloom water to brewer's spent grain extract
310 At the start of the experiment, Williston Lake water had an average phytoplankton
311 density of 420,000 cells/mL with the dominant taxa belonging to Cyanophyta, primarily
312 *Raphidiopsis raciborskii* (88%), followed by *Aphanocapsa sp* (5%), *Microcystis aeruginosa*
313 (2%), and *Microcystis wesenbergii* (1%). Less than 5% of the community consisted of
314 Chrysophyta, Cryptophyta, Chlorophyta, and unidentified flagellates (<10 µl) (Fig. 7). Exposure
315 to 250 mg/L BSG extract resulted in significantly lower total cell densities on days two and four
316 compared to control flasks (log transformed; $p<0.01$[day2], $p<0.001$[day4]; Fig. 7).
317 Phytoplankton community composition also shifted significantly in the 250 mg/L BSG extract
318 treatment relative to controls (PERMANOVA; $F=6.88$ $p<0.05$; Fig. 7). By days two, four, and
319 six, *R. raciborskii* concentrations in the 250 mg/L treatment were 22%, 10%, and 7% relative to
320 controls (Fig. 7). By day six, the phytoplankton community in the 250mg/L treatment had shifted
321 from Cyanophyta dominated to Chlorophyta dominated (Fig. 7). In comparison, lake water with
322 the treatment of 25mg/L BSG extract showed elevated total cell counts throughout the
323 experiment compared to controls, and the relative proportion of Cyanophyta remained consistent
324 with relatively (Fig. 7). Pigment analysis on day six supported these trends. Cultures treated with
325 250 mg/L BSG extract exhibited significantly higher chlorophyll *b* concentrations by day 6
326 (ANOVA Tukey; $F=595$ $p<0.001$). Total chlorophyll-a declined in the control treatment over the
327 six days, going from an average of 68 µg/L to 14 µg/L (Fig. 8).

328 Nutrient analysis of the BSG extract revealed elevated levels of PO_4^{3-} (0.361 mg/L),
329 NH_4^+ (9.61 mg/L), and a low pH (5.31) (Table 1). Based on the nutrient analyses of BSG extract
330 and lake water, the estimated starting concentration for the 25 mg/L (0.1% v/v) and 250 mg/L
331 (1% v/v) BSG extract treatments were 0.249 and 0.333 mg/L NH_4^+, 0.999 and 0.992 mg/L NO_3^-,
332 and 0.0913 and 0.0937 mg/L PO_4^{3-}, respectively. By day 6, NO_3^- and PO_4^{3-} concentrations in
333 flasks treated with 250 mg/L BSG extract were significantly higher (1 mg/L NO_3^- and 0.6±0.07
334 mg/L PO_4^{3-}) than in control flasks (<0.2 mg/L NO_3^- and 0.04±0.01 mg/L PO_4^{3-}) (ANOVA;
335 $p<0.01$; Table 1).

336 ## 4. Discussion

337 ### 4.1 Exposure of microalgae to brewer's spent grain, *Sargassum*, and barley extract

338 These experiments demonstrate that brewer's spent grain (BSG) may serve as a selective
339 and effective control method for harmful algal blooms (HABs). Specifically, BSG reduced
340 growth of toxin-producing cyanobacteria *M. aeruginosa* (Fig. 3), and dinoflagellate *K. brevis*
341 (Fig. 3), while having no effect on the growth of non-toxic diatom *P. tricornutum* (Fig. S1) and
342 chlorophyte *T. obliquus* (Fig. S2). Similarly, other studies have found species of diatoms and
343 green algae to not be impacted or promoted by barley straw (Ferrier et al., 2005; Świerk et al.,
344 2025). These results suggest BSG's potential as a targeted treatment option for managing
345 harmful algal species, without disrupting beneficial or benign phytoplankton. To further explore
346 the observed effects and mode of action of the inhibitory compounds, cultures were also exposed
347 to the purified flavonoid: luteolin, as a positive control. Flavonoids are a class of phenolic
348 compounds widely distributed in plants that are known for their antioxidative properties (Dias et
349 al., 2021). Although luteolin has not previously been identified as a major flavonoid in BSG or
350 *Sargassum*, it has previously been shown to inhibit growth of *M. aeruginosa* through disruption

of photosystem II (PSII) (An et al., 2022; Guo et al., 2025; Huang et al., 2015; Mecina et al., 2017; Xiao et al., 2014). Like BSG, 6 mg/L luteolin inhibited *K. brevis*, with no significant impact on *P. tricornutum* and *T. obliquus* (Fig. 4B, S1 and S2). To our knowledge, this is the first report demonstrating that luteolin has no measurable effect on the growth of chlorophyte or diatom. Notably, luteolin also did not alter bacterial concentrations in *K. brevis* cultures (Fig. 5), unlike BSG, suggesting that microbial interactions may play a unique role in the algicidal activity of BSG in *K. brevis*.

Previous studies have reported similar allelopathic effects of plant extracts on controlling cyanobacteria and other harmful algae, with polyphenols being an important category of control chemicals (Chen et al., 2019; Gao et al., 2020; Nakai et al., 2000; Tang and Gobler, 2011; Wang et al., 2007; Xiao et al., 2014; Zerrifi et al., 2018; Zhao et al., 2019). When comparing the total phenolic content (TPC) quantified in the three plant extracts, barley straw extracts contained the highest TPC per mg/L organic matter, while BSG and *Sargassum* had lower, comparable concentrations of TPC per mg/L organic matter. However, growth inhibition of *M. aeruginosa* and *K. brevis* did not correspond directly with TPC concentrations. For example, despite the higher TPC in barley straw compared to BSG, it did not exhibit stronger growth inhibition of *M. aeruginosa* (Fig. 3). Additionally, *Sargassum* extract had similar TPC concentrations to BSG, but it did not inhibit *M. aeruginosa* growth (Fig. 3). This suggests that either there are other inhibitory compounds present or that inhibition is driven by specific phenolic compounds, rather than total concentrations. Other studies have found similar results suggesting that the specific chemical composition, bioavailability, and environmental water conditions determine whether a specific species of microalgae will be impacted (Li et al., 2023; Liu et al., 2021; Świerk et al., 2025). Different phenolic acids can vary significantly in inhibiting

M. aeruginosa growth, with some compounds effectively killing cells while others have no impact or require higher concentrations to achieve comparable effects (Huang et al., 2015; Li et al., 2023; Verni et al., 2020). For example, Li et al (2023) compared the inhibitory effects of 6 purified phenolic acids on *M. aeruginosa* and found Caffeic acid impacted growth at 5.8 mg/L while its structural analogs (cinnamic acid and ferulic acid) exhibited weak inhibitory effects or promoted growth.

The chemical space of highly aromatic compounds mapped from FT-ICR-MS data, demonstrated clear differences between BSG, Barley, and *Sargassum* extract (Fig. 1). BSG has highly reduced and low oxygen compounds not present in Barley or *Sargassum*. This is likely due to broken down lignin from the heating of the barley husks during the brewing process. Alternatively, Sargassum has unique compounds which are reduced and highly oxidized which matches the expected phlorotannins (Fig. 1). One of the dominant polyphenol groups in brown algae like *Sargassum*, is phlorotannins (Catarino et al., 2023; Cotas et al., 2020; Powers et al., 2019). Phlorotannins have been shown to inhibit the growth of *Karenia mikimotoi*, but no published studies to date have investigated their impact on *Microcystis spp.* (Nagayama et al., 2003). This variation in polyphenol profiles among plant sources may explain the differential algal growth responses observed in this study (Fig. 1, Fig. 3, and Table S1). Additionally, because these experiments used 100% H_2O to extract compounds from the plant material, the resulting profile may differ from that obtained with solvents such as MeOH or acetone. Future research should compare and quantify how different extraction solvents affect the release of phenolic compounds (Świerk et al., 2025). Further research is needed to isolate and identify the specific algicidal compounds in BSG to better understand these different responses between

plant sources, and compare extraction methods since these can impact the phenolic acids and flavonoids released from the particles (Birsan et al., 2019; Meneses et al., 2013).

Many allelochemicals, including polyphenols, have been well documented to interfere with photosystem II (PSII) activity in *M. aeruginosa* (Chen et al., 2019; Huang et al., 2015; Zhao et al., 2019). The F_v/F_m represents the maximum efficiency of PSII photochemistry in cells and a decrease in F_v/F_m has been associated with cellular stress when exposed to biotic and abiotic factors. In this study, both BSG and barley straw extracts at 250 mg/L significantly reduced F_v/F_m values in *M. aeruginosa* within 48 hours (Fig. 4C), indicating early photosynthetic stress. In contrast, 250 mg/L *Sargassum* extract also reduced PSII efficiency after 48 hours but this did not result in growth effects over the eight days (Fig. 4C), suggesting *M. aeruginosa* was able to recover from the initial stress and it was not a high enough dose to lead to apoptosis. Additionally, 50 mg/L doses of BSG and barley straw extracts significantly increased F_v/F_m values in *M. aeruginosa* within 48 hours (Fig. 4C). This could mean a lower dose provides enough nutrient or other compound supplementation to relieve stress and enhance photosystem II efficiency. *P. tricornutum* and *T. obliquus*

The dose-dependent effects of BSG on *M. aeruginosa* growth and PSII efficiency, coupled with the absences of such a response to *Sargassum* extract, suggest that BSG's growth inhibition is primarily driven by its allelochemicals (Fig. 2 and 3). In contrast, while BSG also reduced *K. brevis* growth, the response was not dose-dependent and coincided with increased bacterial abundance (Fig. 3, 6 and S3). This pattern suggested that the inhibition of K. brevis may result from indirect biotic interactions, such as bacterial competition, rather than direct allelochemical toxicity.

418 It is difficult to compare allelopathic effects across studies due to differences in
419 methodology, like variable extraction methods and duration. Most studies estimated g/L doses
420 based on the mixture of dried powder mass/volume of DI water, even though the studies agitate
421 the mixture under different conditions and for various amounts of time ranging from one hour to
422 one week. This study standardized extracts based on dry organic matter content, but the amount
423 of organic matter and phenolic compounds released into water can vary considerably across plant
424 types and extraction protocols. Notably, this study used water extraction and water is a relatively
425 inefficient solvent for phenolic extraction. For instance, studies using rice husks, argued similar
426 concentrations were needed to that of barley straw to inhibit growth of *M. aeruginosa* using H_2O
427 extracts (2-10 g/L), but significantly lower concentrations were needed when rice husks were
428 extracted in MeOH (0.01-10 mg/L) (Park et al., 2006; Su et al., 2014). Future studies could use
429 MeOH, ethanol, or acetone, to extract much higher volumes of phenolic compounds from BSG
430 (Socaci et al., 2018). Ultimately, while 100-250 mg/L doses of BSG (0.4-1% v/v extract) were
431 effective in laboratory settings, these concentrations are impractical for direct application to
432 natural water bodies. Future efforts should focus on optimizing extraction methods to
433 concentrate active algicides, reducing the required application volume although this will increase
434 the cost of the control technology.

435 4.2 Exposure of *Karenia brevis* to brewer's spent grain with and without antibiotics

436 To further understand whether the growth inhibition of *K. brevis* by BSG extract was due
437 to chemical compounds or associated bacterial activity, antibiotics were used to suppress
438 bacterial growth in cultures. Control treatments with and without antibiotics resulted in similar
439 *K. brevis* growth rates (Fig. 4), indicating that the antibiotics themselves did not affect algal
440 growth. Furthermore, 6 mg/L luteolin inhibited *K. brevis* growth equally in the presence and

absence of antibiotics (Fig. 4B), confirming that the antibiotics did not interfere with the action of this flavonoid. In BSG-treated cultures, however, the antibiotics were less effective at suppressing bacterial proliferation. Bacterial abundance remained similar to controls for the first 24 hours in the 200 mg/L BSG treatment and for 48 hours in the 50 mg/L treatment, but were significantly higher after this (Fig. 5). This suggested that multiple doses of antibiotics may be required for future experiments extending beyond 48 hours. Despite this limited bacterial control, antibiotic treatment had an impact on *K. brevis* response to BSG extract. In the absence of antibiotics, 200 mg/L BSG inhibited growth of *K. brevis* through the experiment but when antibiotics were included, BSG had no effect on algal growth compared to the control (Fig. 4A). This supports the idea that biotic interactions, play a central role in BSG-induced inhibition of *K. brevis.* However, variability within treatments complicates this interpretation. In both 50 mg/L and 200 mg/L BSG treatments, some flasks with high bacterial abundance still supported *K. brevis* growth, while others with lower bacterial levels showed growth inhibition. These inconsistencies suggest that the effect may not be due to total bacterial abundance alone, but possibly due to the enrichment of specific bacterial taxa that negatively impact *K. brevis* or metabolic interactions.

To further investigate this relationship, 16S rRNA sequencing was performed to characterize the bacterial communities in flasks on day four of the experiment. Bacterial community composition varied substantially across treatments and with antibiotic use. The overall Shannon diversity index of bacteria was not impacted using antibiotics, except in 50 mg/L BSG extract without antibiotics where it was significantly lower (Table S2). These results differ from other studies which reported reductions to diversity following antibiotic exposure (Cao et al., 2019).

Notably, flasks with 50 mg/L and 200 mg/L BSG extract (without antibiotics), which also experienced the highest *K. brevis* mortality, had elevated levels of *Alteromonas* spp. (Fig. 6). These findings align with those of Sun et al. (2022), who observed blooms of *Alteromonas* and *Halomonas* coinciding with *Karenia mikimotoi* die-offs following carbon enrichment (Sun et al., 2022). Similarly, *Alteromonas* species have been reported to inhibit *K. mikimotoi* growth directly (Zheng et al., 2018). As BSG extract is a rich source of organic carbon (Table 1), it likely stimulated heterotrophic bacterial growth, favoring taxa such as *Alteromonas* that may exert antagonistic effects on *K. brevis*. Antibiotics diminished the enrichment of these taxa, supporting the biotic nature of the inhibition.

These results contrast with prior laboratory experiments showing consistent inhibition of dinoflagellates by *Sargassum* extracts in axenic cultures (Wang et al., 2012, 2007). Several factors may account for the differences, including insufficient *Sargassum* extract doses in this study, different in phenolic compound composition between *Sargassum* and BSG, or species-specific tolerance of *K.brevis*. It is clear from these results that the flavonoid, luteolin, can inhibit the growth of *K. brevis* and more research is needed to look at the impact of other flavonoids as well as why high bacterial abundance led to inconsistent inhibition of *K. brevis* growth.

Ultimately, this study demonstrates that while luteolin clearly inhibits *K. brevis*, the BSG extract inhibition observed is largely biotic, mediated by shifts in microbial community composition, particularly the enrichment of bacteria like *Alteromonas*. These results highlight the need for additional research to explore microbial-algal interactions and to identify specific bacterial taxa and metabolites responsible for growth suppression. Since microbial communities can shift with algal growth stage and physiology (Li et al., 2019; Sun et al., 2022), future

experiments should include time-series microbial sampling to capture dynamic interactions over the course of treatment.

4.3 Exposure of *Raphidiopsis raciborskii* bloom water to brewer's spent grain extract

A dosage of 250 mg/L BSG extract (1% v/v) had a significant impact on phytoplankton community composition and pigment profiles in the Williston Lake water microcosms over the six-day experiment (Fig. 7 and 8). In particular, the concentration of *R. raciborskii* declined, while chlorophytes and the pigment chlorophyll b concentration increased over the six days compared to the control treatment (Fig. 7 and 8). This shift is consistent with previous findings in lake water treated with decomposing barley straw, where cyanobacteria were more strongly affected compared to diatoms or green algae (El-Moneem et al., 2012; Ferrier et al., 2005).

Alternatively, a lower dosage of 25 mg/L BSG extract (0.1% v/v) had no significant impact on either phytoplankton community composition or pigment composition compared to the controls over the same period (Fig. 7 and 8). After 2 days, the 25 mg/L BSG treatment had an increase in cyanobacteria abundance relative to the control, although the difference was not significant. The 25 mg/L dose was likely not a sufficient concentration of allelochemicals to suppress *R. raciborskii* (Table 1).

These results are interesting as Lusty and Gobler (2020) found *R. raciborskii* to be most resistant to hydrogen peroxide treatment. Since barley straw and flavonoids are thought to act via similar mechanisms, the generation of reactive oxygen species (ROS) and induction of oxidative stress, further work is needed to evaluate if other common cyanobacteria, like *Microcystis*, might be more sensitive to lower doses of BSG extract (Mecina et al., 2019, 2017; Xiao et al., 2014). Importantly, at both these low concentrations, the added extracts would not measurably alter ambient nutrient levels (mg/L), indicating that the observed patterns were likely not due to

nutrient enrichment and the final day 6 chemistry shifts were primarily due to algal cell death (Table 1).Still, these results emphasize the importance of dosage in applying BSG extract as a mitigation technique in the field, as inadequate dosing could inadvertently promote bloom formation rather than suppress it and the added nutrient load introduced by the extract is not desirable for long-term HAB prevention.

5. Conclusion

This study demonstrated the potential use of brewer's spent grain (BSG) as a control strategy for harmful algal blooms (HABs). Laboratory experiments revealed that BSG extract inhibited the growth of toxin-producing dinoflagellate, *Karenia brevis*, and cyanobacteria species, *Microcystis aeruginosa,* without impacting the growth of the diatom, *Phaeodactylum tricornutum,* and the chlorophyte, *Tetradesmus obliquus* (Fig. 3, S1, and S2). However, results suggest that bacterial growth or the proliferation of a specific bacterial strain, was likely responsible for growth inhibition in *K. brevis* cultures rather than direct allelopathic effects (Fig. 4 and 5). In microcosms using water experiencing *Raphidiopsis raciborskii* bloom, exposure to 250mg/L of BSG extract caused a collapse in *R. raciborskii*, and a subsequent shift in dominance toward chlorophytes. Despite these promising results, the extract contained PO_4^{3-} and NH_4^+ which may stimulate blooms if this technique is used in the field (Table 1). Additionally, concentrations of 250 mg/L BSG extract are logistically impractical for large-scale field use. Therefore, to make BSG a viable management tool, the active allelochemicals would need to be isolated and concentrated. This could potentially be achieved using high-organic solvents, such as methanol or ethanol, though such methods would increase the cost of implementation. Future research should explore the economic feasibility of extracting the inhibitory compounds for BSG, as well as investigate alternative applications for this waste byproduct. Additionally, more

research is needed to evaluate the potential of luteolin as a targeted control strategy, and how best to disperse the compound in HAB-impacted waterbody.

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Table 1. Concentration of nutrients in controls and treatments after six days of exposure to filtrates from brewer's spent grain (BSG).

Day	n	Treatment	pH	NH_4^+ (mg/L)	NO_3^- (mg/L)	NO_2^- (mg/L)	PO_4^{3-} (mg/L)	CO_3^{2-} (mg/L)
0	1	Lake water	7.34	0.24	1	0.01	0.091	NA
	1	BSG extract	5.31	9.61	0.2	<0.01	0.361	NA
6	2	Control	8.37±0.28	0.03±0.02	<1	<0.01	0.04±0.01	55.47±3.28
	2	25 mg/L BSG extract	8.23±0.19	0±0.01	<1	<0.01	0.07±0.02	54.97±1.03
	2	250 mg/L BSG extract	7.76±0.11	0.02±0.02	1	<0.01	0.5±0.14	64.4±5.89

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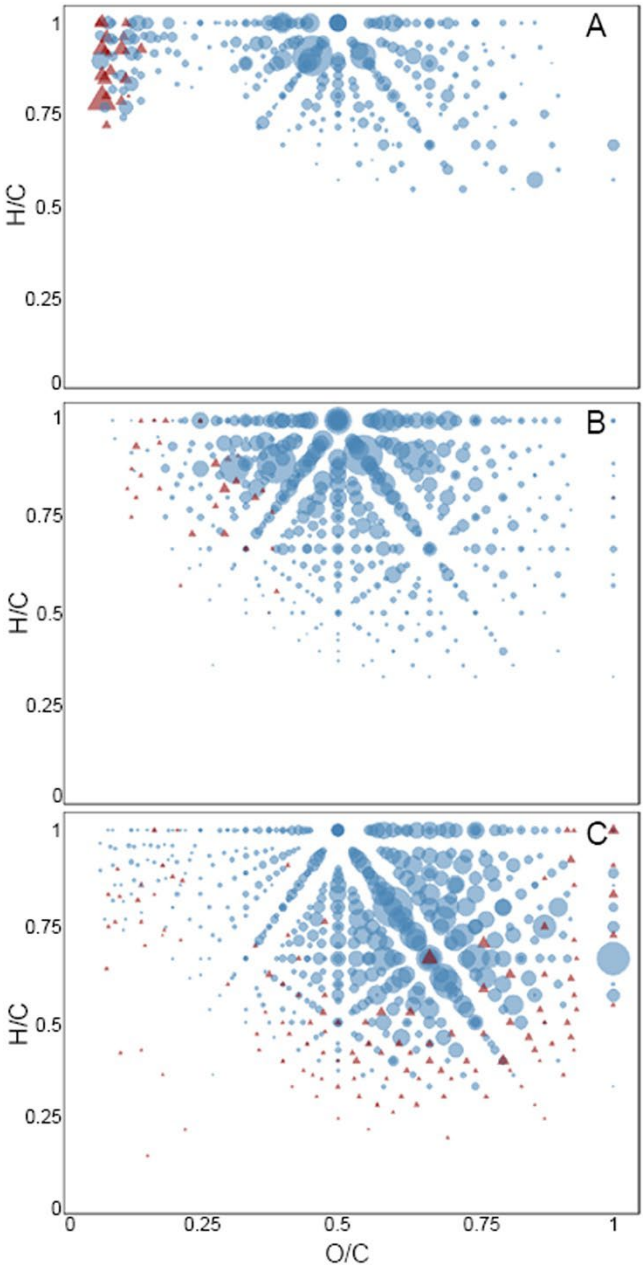
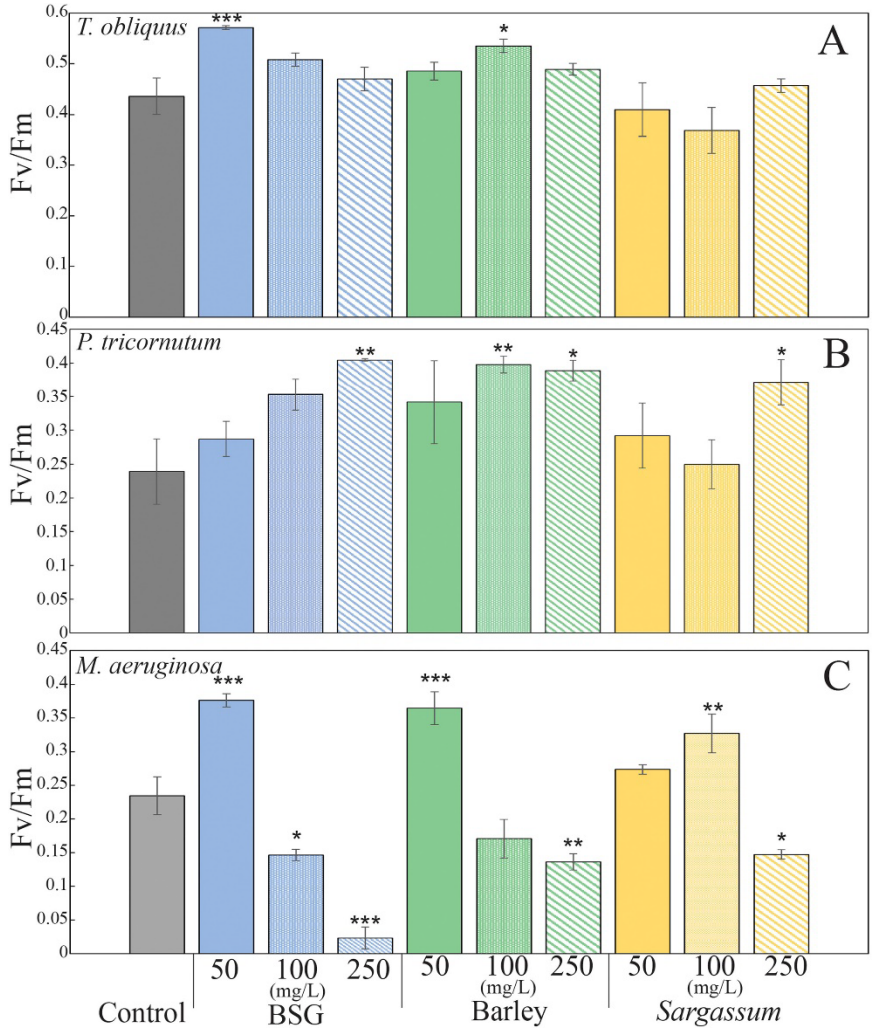
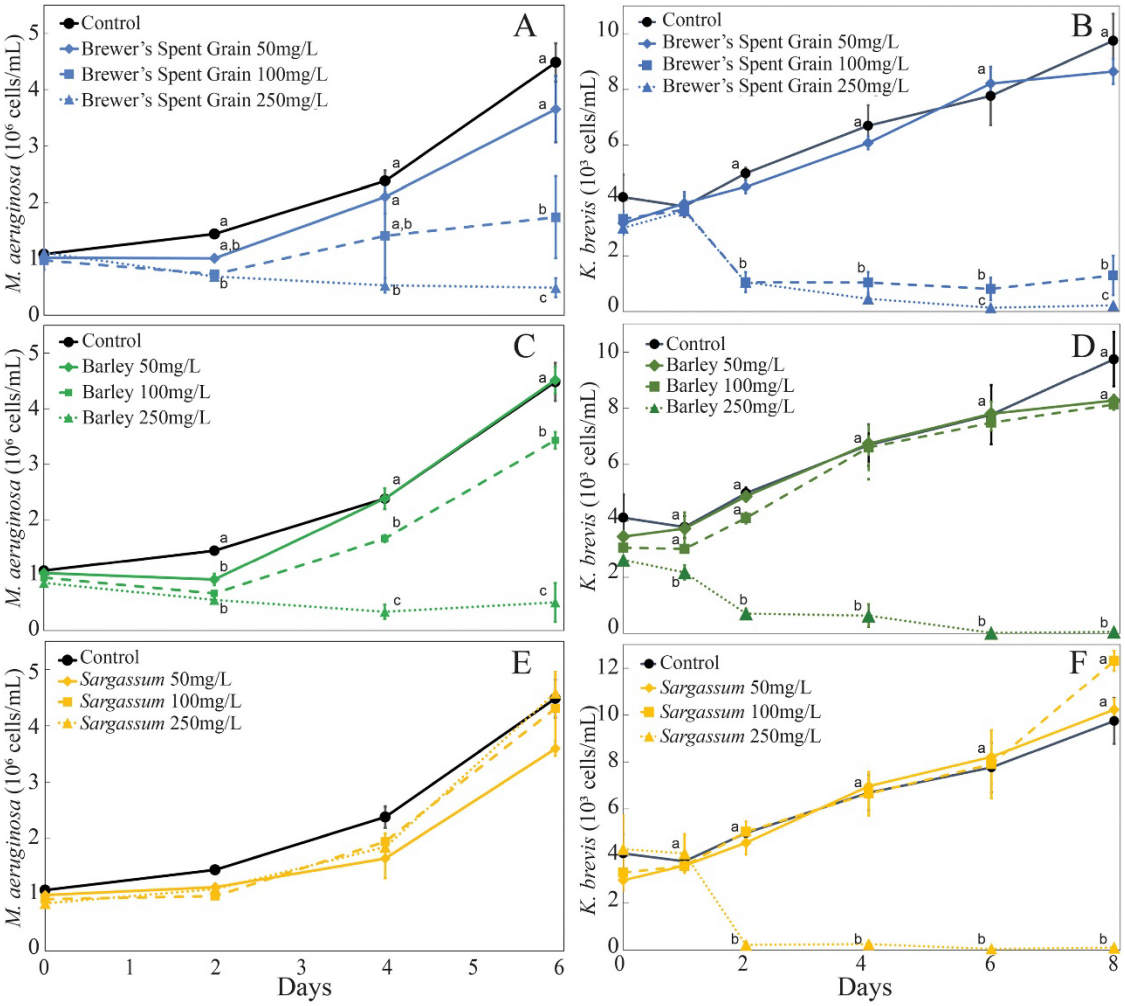
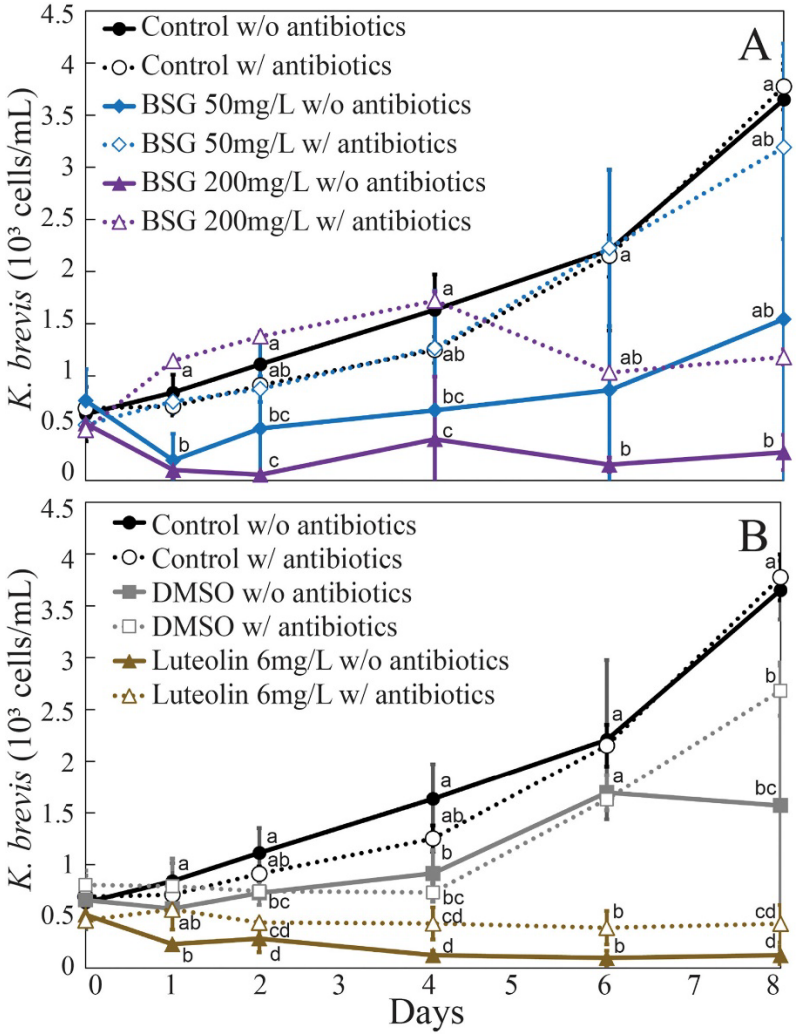
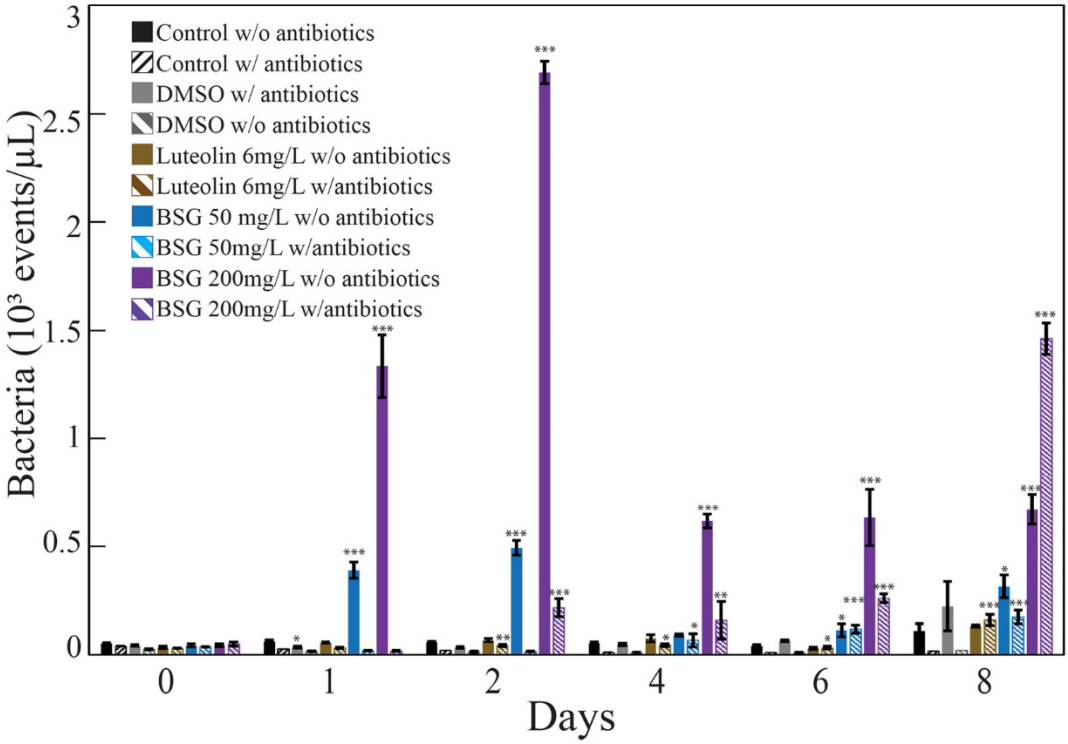
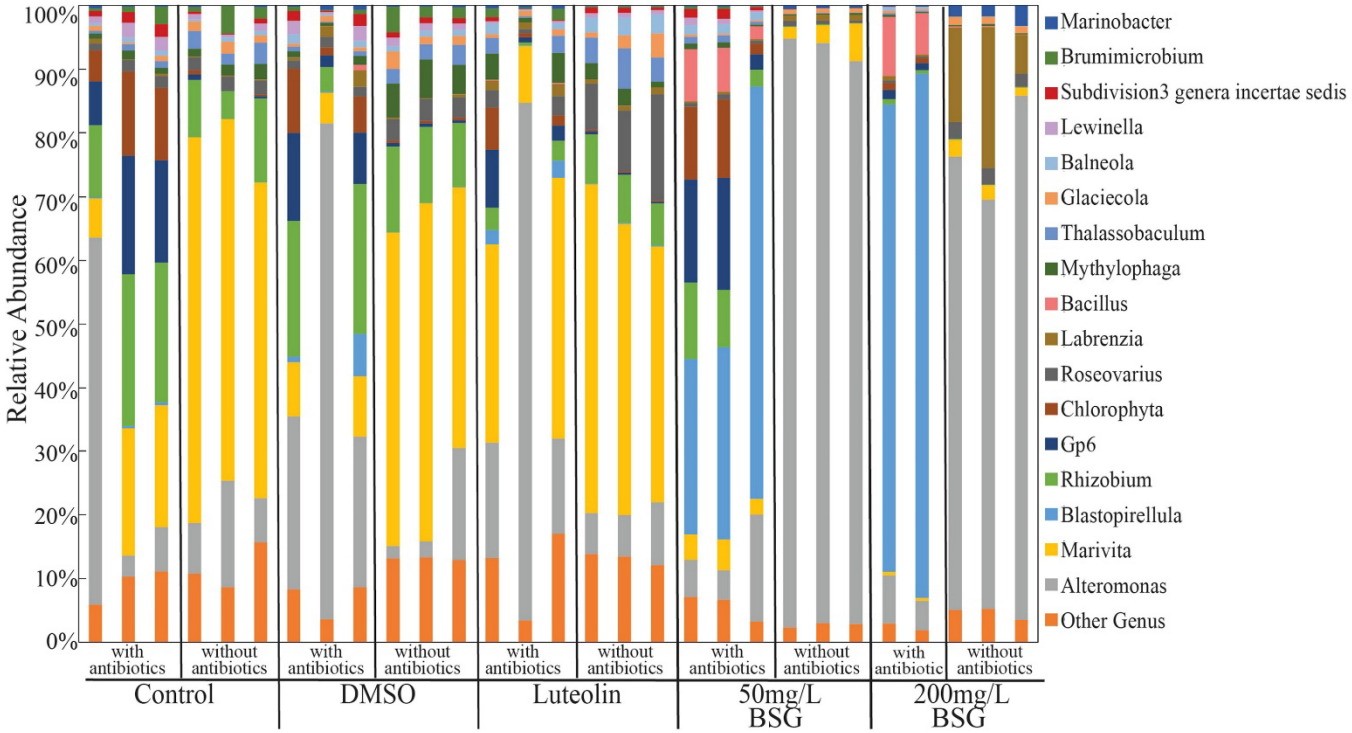

A
B
C
H/C
O/C
0
0.25
0.5
0.75
1

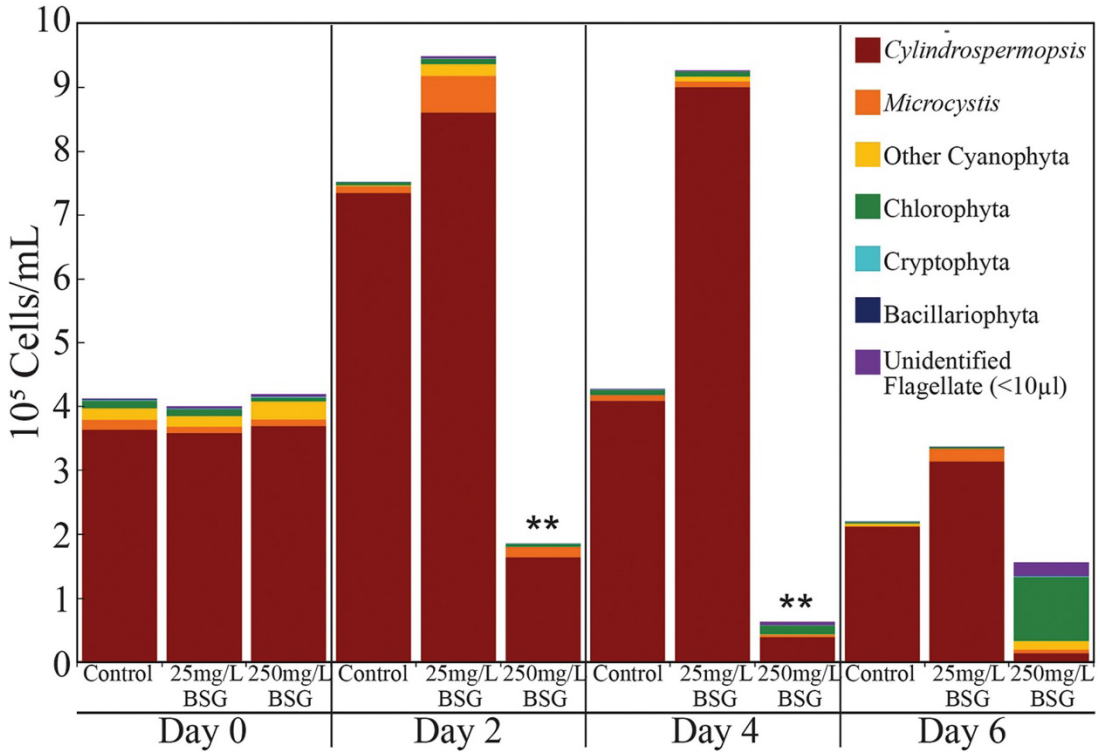

T. obliquus
A
P. tricornutum
B
M. aeruginosa
C
Fv/Fm
Control
50
100
(mg/L)
250
BSG
50
100
(mg/L)
250
Barley
50
100
(mg/L)
250
Sargassum

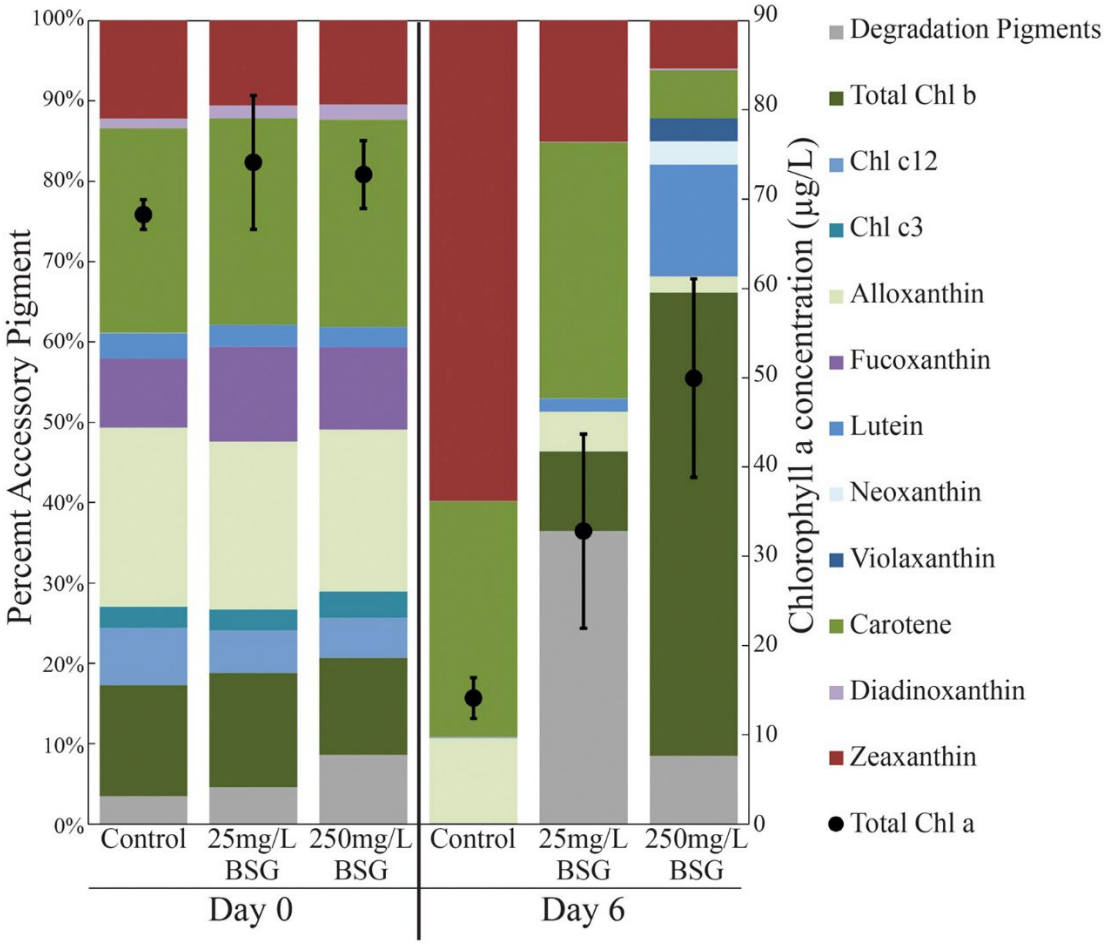

A
Control
Brewer's Spent Grain 50mg/L
Brewer's Spent Grain 100mg/L
Brewer's Spent Grain 250mg/L
M. aeruginosa (10⁶ cells/mL)
B
K. brevis (10³ cells/mL)
C
Barley 50mg/L
Barley 100mg/L
Barley 250mg/L
D
E
Sargassum 50mg/L
Sargassum 100mg/L
Sargassum 250mg/L
F
Days


A
Control w/o antibiotics
Control w/ antibiotics
BSG 50mg/L w/o antibiotics
BSG 50mg/L w/ antibiotics
BSG 200mg/L w/o antibiotics
BSG 200mg/L w/ antibiotics
K. brevis (10³ cells/mL)
B
Control w/o antibiotics
Control w/ antibiotics
DMSO w/o antibiotics
DMSO w/ antibiotics
Luteolin 6mg/L w/o antibiotics
Luteolin 6mg/L w/ antibiotics
K. brevis (10³ cells/mL)
Days


Control w/o antibiotics
Control w/ antibiotics
DMSO w/ antibiotics
DMSO w/o antibiotics
Luteolin 6mg/L w/o antibiotics
Luteolin 6mg/L w/antibiotics
BSG 50 mg/L w/o antibiotics
BSG 50mg/L w/antibiotics
BSG 200mg/L w/o antibiotics
BSG 200mg/L w/antibiotics
Bacteria (10³ events/μL)
Days
0
0.5
1
1.5
2
2.5
3
0
1
2
4
6
8


Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
with antibiotics
without antibiotics
Control
DMSO
Luteolin
50mg/L BSG
200mg/L BSG
Marinobacter
Brumimicrobium
Subdivision3 genera incertae sedis
Lewinella
Balneola
Glaciecola
Thalassobaculum
Mythylophaga
Bacillus
Labrenzia
Roseovarius
Chlorophyta
Gp6
Rhizobium
Blastopirellula
Marivita
Alteromonas
Other Genus


10
9
8
7
6
5
4
3
2
1
0
10^5 Cells/mL
Cylindrospermopsis
Microcystis
Other Cyanophyta
Chlorophyta
Cryptophyta
Bacillariophyta
Unidentified Flagellate (<10µl)
**
**
Control
25mg/L BSG
250mg/L BSG
Day 0
Day 2
Day 4
Day 6


Percemt Accessory Pigment
Chlorophyll a concentration (µg/L)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
90
80
70
60
50
40
30
20
10
0
Control
25mg/L BSG
250mg/L BSG
Control
25mg/L BSG
250mg/L BSG
Day 0
Day 6
Degradation Pigments
Total Chl b
Chl c12
Chl c3
Alloxanthin
Fucoxanthin
Lutein
Neoxanthin
Violaxanthin
Carotene
Diadinoxanthin
Zeaxanthin
Total Chl a